The catabolism of glucose by the thermoacidophilic archaebacterium Thermoplasma acidophilum

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THE CATABOLISM OF GLUCOSE BY THE THERMOACIDOPHILIC ARCHAEBACTERIUM THERMOPLASMA ACIDOPHILUM

Submitted by Nigel Budgen
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Two roads diverged in a wood, and I-
I took the one less travelled by,
And that has made all the difference.

Robert Frost

from The Road Not Taken

CONTENTS

SUMMARY

It has been found that the thermoacidophilic archaebacterium, Thermoplasma acidophilum, can metabolise glucose via a modified Entner-Doudoroff pathway involving non-phosphorylated intermediates. Pyruvate and glyceraldehyde are the first products, the glyceraldehyde then being further metabolised to a second molecule of pyruvate via 2-phosphoglycerate. Intermediates of the pathway have been identified by enzymic analysis or by thin-layer chromatography and the individual enzymes involved have been assayed and some of their kinetic parameters determined. Radiorespirometeric analysis of Tp. acidophilum grown on various ^{14}C-labelled glucoses confirmed the existence of an Entner- Doudoroff pathway in growing cells. This is also supported by the ^{14}C-labelling patterns of acetic acid, which was excreted when cells were grown on various ^{14}C-glucoses.

Glyceraldehyde reductase [EC 1.1.1.72] activity was identified in cell extracts of Tp. acidophilum and Sulfolobus acidocaldarius. Kinetic parameters are presented with evidence which suggests these activities provide glycerol for the synthesis of ether lipids.

An acetyl-CoA synthetase [EC 6.2.1.13] activity was found in cell extracts of Tp. acidophilum which is shown to be ADP dependant. Its kinetic parameters are presented.

Glucose dehydrogenase [EC 1.1.1.47],from Tp.acidophilum, the first enzyme of the pathway, was purified. The enzyme possessed dual cofactor specificity, requiring either NAD^+ or $NADP^+$, and showed oxidation of either glucose or galactose.

Comparisons are made with the pathways and enzymes of glucose catabolism found in other archaebacteria, eubacteria and eukaryotes.

CHAPTER 1.

INTRODUCTION

1.1 The archaebacteria

In the late 1970's a radically new concept of three rather than two evolutionary kingdoms challenged the accepted dogma. The concept of the archaebacteria, as this third additional kingdom came to be known, has revolutionised the views on the evolutionary relationships between the eubacteria and eukaryotes as well of those about the common ancestor of all forms of life.

It was Carle Woese and George Fox who were first to recognise this fundamental concept. It became apparent after analysis of the sequences of 16S and 18S ribosomal RNA of a number of organisms. rRNA was used for measuring phylogenetic relationships since it has a constant function, is universal in distribution, is easily isolated, and is a relatively large molecule containing highly and moderately conserved sequences across large phylogenetic distances (Woese, 1985). It is argued that rRNA sequencing can provide an accurate measure of distant and close phylogenetic relationships. Partial sequencing of the 16S and 18S rRNA was achieved by digesting with ribonuclease T_1 separating the oligonucleotides and determining their sequence. The resulting sequenced oligonucleotides, which are characteristic of an organism, when quantitatively compared with another set of sequenced oligonucleotides from another organism provide a measure of the phylogenetic

relationship. Using coefficents based on the fraction of common bases between the two sets of oligonucleotides of six or greater bases, Fox et al. (1977) built up a phylogenetic tree. This analysis and interpretation led Woese & Fox (1977) to propose the existence of at least three kingdoms, the eubacteria (true bacteria), the eukaryotes and the archaebacteria.

The archaebacteria can be divided into three phenotypes sulphur dependant, halophilic and methanogenic (reviewed by Woese & Olsen, 1986; Fewson, 1986). The sulphur dependant archaebacteria (Stetter & Zillig, 1985) are all found in thermal environments (55-110°C) being either aerobic or anaerobic. Many of them are thermoacidophiles growing at low pH values (pH 1-2) such as Thermoplasma acidophilum and Sulfolobus acidocaldarius. Some are chemolithotrophic capable of reducing or oxidising sulphur but other members are heterotrophs.

The halophiles require extremely high concentrations of NaCl, some growing in saturated salt solutions (5.2M). This group is divided into the classical and the alkalophilic halophiles. The latter are so named since they grow optimally at pH 9 to 10 (Kushner,1985).

The methanogens are strict anaerobes which reduce CO_2 with H_2 to methane as the sole energy providing process (Balch et al., 1979; Whitman, 1985).

Each of the groups of the archaebacteria exist in extreme ecological niches, which may be similar to conditions thought to have prevailed on earth $3\text{-}4X10^9$ years

ago. The name archaebacteria originated from such an observation suggesting they are of a primeval nature (Woese and Fox 1977), although this concept is still a matter of contention.

The complete sequence of 16S/18S rRNA molecules of various methanogenic, halophilic, sulphur dependant archaebacteria, and also some eubacteria and eukaryotes, have been determined (referenced in Woese and Olsen, 1986). The data support the concept of the three kingdoms and provide further evidence of two main divisions in the archaebacteria (figure 1.). The first division is composed of the thermophilic sulphur-dependant archaebacteria, excluding Thermococcus celer and Tp. acidophilum. The second is composed of the methanogens and extreme halophiles including Tc. celer and Tp. acidophilum.

Hybridisation homologies of total rRNA of 17 species of archaebacteria with various nitrocellulose bound DNA's supported in principle the two divisions (Klenk et al.,1986). The phylogenetic tree constructed (figure 2) does differ slightly in a few details, suggesting that Thermococcales form a third division in the archaebacterial kingdom.

Besides rRNA sequencing a number of other features have been used to determine the phylogenetic organisation, such as 5S rRNA secondary structure (Fox et al.,1982; Wolters & Erdmann, 1986), the structure of DNA-dependant RNA polymerase (Zillig et al.,1985) and ribosome morphology (Lake et al.,1984; 1985). In all a relationship between the sulphur-dependant archaebacteria and eukaryotes is

suggested with the eubacteria arising from the halophilic-methanogenic branch. Lake et al. (1984; 1985) and Lake (1986) go further to suggest that there are four separate kingdoms, the sulphur dependant archaebacteria forming the 'eocytes', the extreme halophiles and eubacteria the 'photocyta' and the archaebacteria being formed only by the methanogens. This proposal has been critically reviewed by Woese & Olsen (1986).

Characteristic biochemical features possessed by the archaebacteria further reinforce their distinct position. The membrane lipids of the archaebacteria are unique consisting of isopranyl ether-linked glycerol lipids with an sn-2,3-glycerol configuration (Langworthy, 1985). The membrane lipids contrast with the sn-1,2-glycerol fatty acyl ester linked lipids of the eubacteria and eukaryotes. The thermoacidophiles and some methanogens possess tetraethers that span the whole membrane forming a lipid monolayer, which in the thermoacidophiles contain cyclopentyl rings (Langworthy, 1985). The cell envelope possessed by the archaebacteria is also unique since it lacks the characteristic eubacterial murein (Kandler & Konig, 1985). Another unique feature are the modifications of the tRNA, which are only found in archaebacterial species (Gupta, 1985).

As well as having unique features the archaebacteria also share fundamental features with the eukaryotes and eubacteria. The archaebacteria are morphologically and structurally similar to the eubacteria. The components of the archaebacterial ribosome share features of the

eubacterial structure (Matheson,1985). The 70S ribosome contains one molecule of 5S, 16S and 23S rRNA (Visentin et al., 1972) and the organisation of these rRNA genes is similar (Hofman et al., 1979). The 16S rRNA of a halophile was found to possess a Shine-Dalgarno sequence (Kagramanova et al., 1982) and closely resembles that of eubacterial 16S rRNA than of the 18S rRNA of eukaryotes (Kagramanova et al., 1982; Gupta et al., 1983). However the whole ribosome is of eubacterial nature, but constructed of eukaryotic like component molecules (Matheson, 1985).

In addition to the unique and eubacterial features the archaebacteria also have eukaryotic features. Introns have been found in the genes coding for tRNA leucine and serine in Sulfolobus solfataricus (Kaine et al., 1983), for tryptophan in Halobacterium volcanni (Daniels et al., 1985) and for 23S rRNA in Desulfurococcus mobilis (Kjems & Garrett, 1985). Histone-like proteins associated with DNA have been found in thermoacidophilic (Searcy & Stein, 1980; Green et al. 1983) and methanogenic (Thomm et al., 1982) archaebacteria. Partial sequence homology between the HTa protein from Tp. acidophilum and the eukaryotic DNA-binding protein has been shown (Searcy & Delange, 1980). Some archaebacterial mRNA's have been shown to have polyadenylated tails at the 3'termini, simular to those found in eukaryotes (Ohba & Oshima, 1983); Oshima et al., 1984). Protein synthesis initiation from mRNA in archaebacteria appears to start with methionyl-tRNA and not with N-formyl methionyl tRNA as in eubacteria (Gupta,

1985). The elongation factor (EF2 found in archaebacteria is polyadenylated by diphtheria toxin as is the eukaryotic EF2. The transcriptional apparatus of archaebacteria shows a 'eukaryotic type' sensitivity to various antibiotics (Bock & Kandler, 1985)

In summary, the archaebacteria constitute a third kingdom of possibly primitive organisms, that possess both unique and common biochemical features when compared with the eubacteria and eukaryotes. They represent a group of organisms which question establisned evolutionary theory. All these features make this group of organisms extremely interesting to study.

1.2. Thermoplasma acidophilum and Sulfolobus sp.

In this section a general description is given of both organisms that were studied in this thesis. Extensive reviews of them are given by Brock(1978) and Stetter & Zillig(1985). The first archaebacterium covered is Thermoplasma acidophilum, the study of which forms the bulk of the research.

The order Thermoplasmales of which there is only one representative, Thermoplasma acidophilum, have the following characteristics; they are regular or elongated cocci that lack cell walls, they are aerobic, obligately heterotrophic growing on yeast extract and glucose, and moderately thermophilic growing between 50°C and 65°C and pH 0.8 to 3.0.

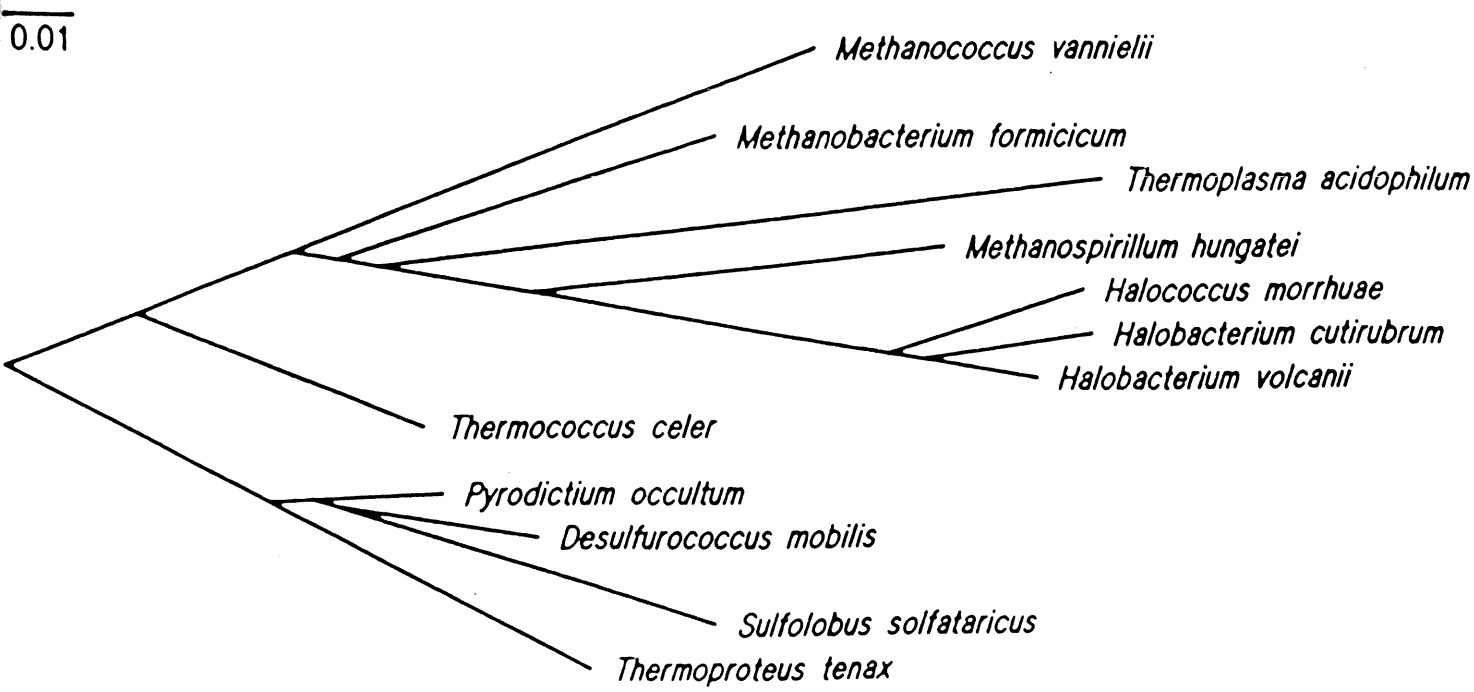
Thermoplasma acidophilum was first isolated by Darland

et al.(1970) from an unusual habitat, a smouldering coal refuse pile in Indianania, USA. At first this curious organism was considered to be a thermophilic mycoplasma but further investigation of its osmotic stability (Belly & Brock, 1972), its membrane lipids (Langworthy et al., 1972), and its 16S rRNA sequence data (Woese et al. 1980; Fox et al., 1980) showed it to be an archaebacterium.

Belly & Brock (1972) carried out an extensive survey to isolate Tp. acidophilum from the natural sulphataric hot springs in Yellowstone park, USA, without success, (but in fact found another archaebacterium Sulfolobus). Ohba & Oshima (1982) claim to have isolated a Thermoplasma sp. from a Japanese hot spring. The organism isolated possessed lipids and an RNA polymerase that are closely related to Tp. acidophilum (Stetter & Zillig, 1985). The presence of primary producers Sulfolobus and Thermoproteales in the only natural environment may provide nutrients for the heterotrophic existence for Thermoplasma.

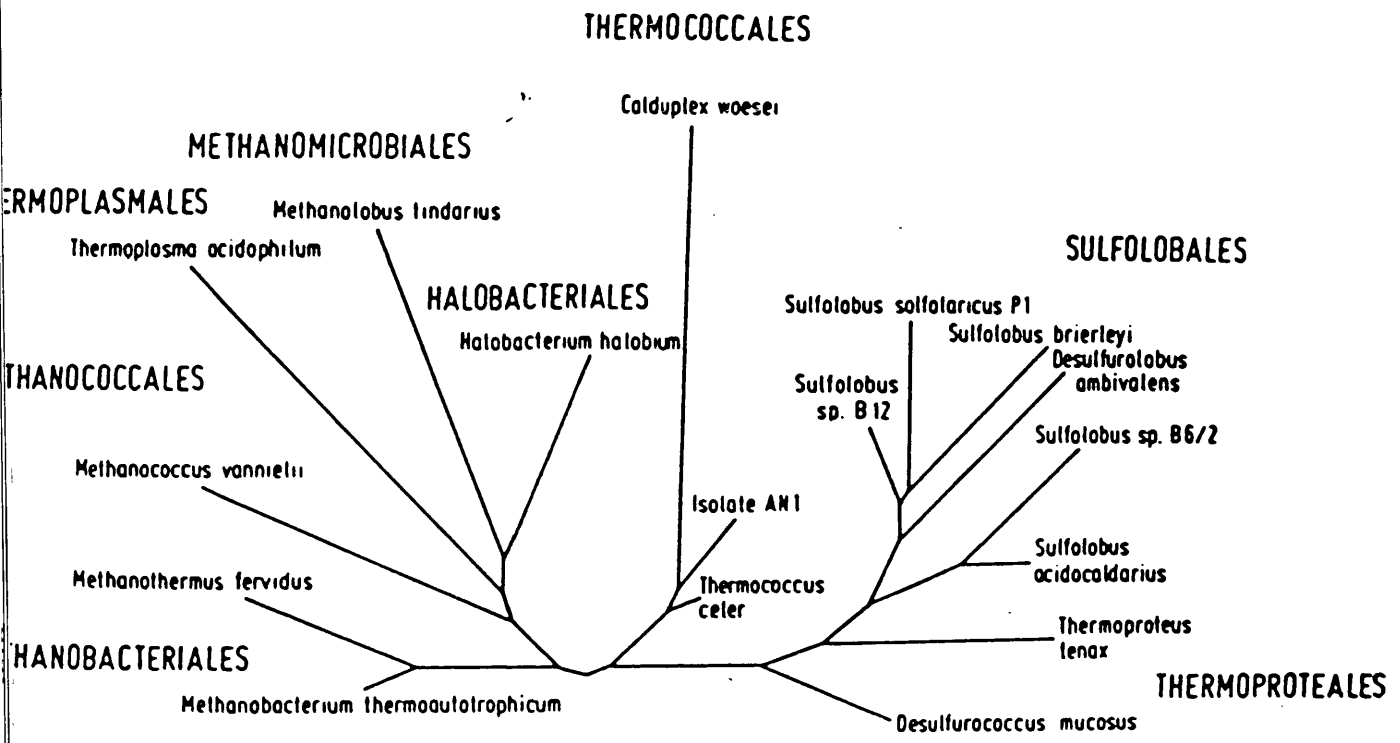
Tp. acidophilum has a DNA base composition of 46% G + C(Christiansen et al., 1975; Searcy & Doyle, 1975) and a genomic size of between $8.4\text{-}10\text{X}10^8$ daltons, which is one of the smallest reported for a free living organism. A basic histone-like protein is associated with its DNA (Searcy & Stein, 1980) which is a eukaryotic characteristic, and which has been partially sequenced by Searcy & Delange (1980). Another eukaryotic-like feature is the possession of an actin-like molecule, which may be involved in a cytoskeletal role. These features and the nature of the

Figure 1. Unrooted phylogenetic tree for the archaebacteria based on 16S rRNA sequences



Complete rRNA sequences were aligned and estimates of sequence divergence (mutations fixed per sequence position) were calculated and used by Woese and Olsen (1986) to infer the phylogenetic tree. The scale bar corresponds to a tree branch lenght of 0.01 mutations fixed per sequence position.

Figure 2. Phylogenetic tree of the archaebacteria based on hybridisation homologies



The tree was constructed by Klenk et al. (1986) from hybridisation homologies between rRNA and nitrocellulose-bound DNAs. The organisms on both sides of the tree have the same mean distance to the vortex at the centre of the tree.

superoxide dismutase (Searcy and Searcy,1981) have lead Searcy to suggest that Tp, acidophilum might be specially related to the eukaryotes (Searcy et al., 1978).

The membrane of Tp. acidophilum contains both diethers and tetraether lipids which are characteristic of the archaebacteria (Langworthy et al., 1972). It also contains a lipopolysaccaride of unique strucure (Mayberry-Carson et al.,1974 ; Smith,1980) and at least one glycoprotein (Young & Hong, 1979). The membrane also contains a respiratory chain composed of cytochrome b and a menaquinone which are capable of reducing oxygen (Searcy & Whatley 1982). Also a sulphate but not proton exporting ATPase was found (Searcy & Whatley,1982). It appears that protons are pumped out by the respiratory assisted export of sulphate, suggesting that energy could only be produced by substrate phosphorylation (Searcy & Whatley,1982)

The internal pH of Tp.acidophilum has been calculated by Hsung & Hang (1975) to be in the region of 6.5 using a labelled marker 5,5-dimethyl 2,4-oxazolidine. However Searcy (1976), using a titrameteric method, found the internal pH to be closer to pH 5.4.

Tp. acidophilum has been shown to be aerobic (Smith et al.1973). This is supported by Searcy & Whatley (1984) who showed that cells incubated for 25min without oxygen lost viability.

In addition, it is an heterotropn requiring yeast extract and glucose for growth. Belly et al. (1973) showed that when grown on 0.025%(w/v) yeast extract under limiting conditions, growth was stimulated by sucrose

glucose, galactose, mannose, and fructose, suggesting that the organism metabolises these compounds. Glycerol, ribose, aspartate, glutamate, glycine and alanine did not stimulate growth. The pathways of catabolism of the compounds in this organism were not known.

The exact nutrient in the yeast extract required by Tp. acidophilum was investigated by Smith et al. (1975). They isolated a peptide Mr 1000 which contained 8-10 amino acids and which was essential for growth. Smith et al. (1975) suggested that the peptide was not a simple nutrient but a functional compound. They proposed a number of roles it could be involved in; a) act as an ion scavenger for trace metals b) protection of the organism from high pH c) involved in ion transport d) in a supply of essential amino acids. The naturally occurring substitue for this compound present in the coal refuse has yet to be identified.

The order Sulfolobales describes organisms with the following characteristics; aerobic irregular cocci capable of both heterotrophic or chemolithoautotrophic growth and oxidising elemental sulphur to sulphate between the temperatures of 50°C-90°C and from pH 1 to 5.5. They are capable of synthesising glycogen and possess a cell envelope composed of a hexagonal array of protein or glycoprotein subunits (Weiss,1974; Michel et al.,1980).

Sulfolobus was first isolated by Brierley (1966) from acidic hot springs in Yellowstone park and was fully described by Brierley & Brierley(1973). Independantly Brock published a full description in 1972 (Brock et

et al.,1972), followed by DeRosa et al.(1974) after isolating another similar organism from Italian solphataric hot springs. When the component patterns of the DNA-dependent RNA polymerases of the Italian and American isolates were compared, the data showed them to be similar but not identical (Zillig et al., 1979;1980). The three isolates were named Sulfolobus acidocaldarius (Brock isolate), Sulfolobus solfataricus (DeRosa isolate) and Sulfolobus brierleyi (Brierley isolate) (Zillig et al.,1980).

The nature of the cell envelope (Weiss, 1974; Michel et al.,1980), the membrane (Langworthy et al.,1974,1982; DeRosa et al.1977), the ribosomes (Fox et al.,1980), the elongation factors EFII (Kessel & Klink,1980) and the RNA polymerase (Zillig et al., 1979,1980) proved that all three Sulfolobus species are typically archaebacterial.

The optimal growth temperatures and pH values of the different isolates differ considerably (Zillig et al.,1980); Sulfolobus acidocaldarius grows heterotrophically at 70°C and pH 2.0 in a medium containing mineral salts and yeast extract. S.solfataricus also grows heterotrophically at 87°C and pH 3.5 on a simple media containing one of the following; glucose, galactose, allolose, xylose, ribose, lactose, maltose or trehalose as the sole source of carbon (DeRosa et al., 1984). S. brierleyi is also capable of growth on glucose (Wood et al.,1987).

Sulfolobus species are capable of chemolithoautotrophic growth by oxidising elemental sulphur to sulphate. Under these conditions, CO_2 serves as the carbon source, being assimilated via a reductive carboxylic acid cycle and not via the Calvin-Benson cycle as usually found in eubacteria (Kandler & Stetter, 1981).

1.3. The catabolism of glucose.

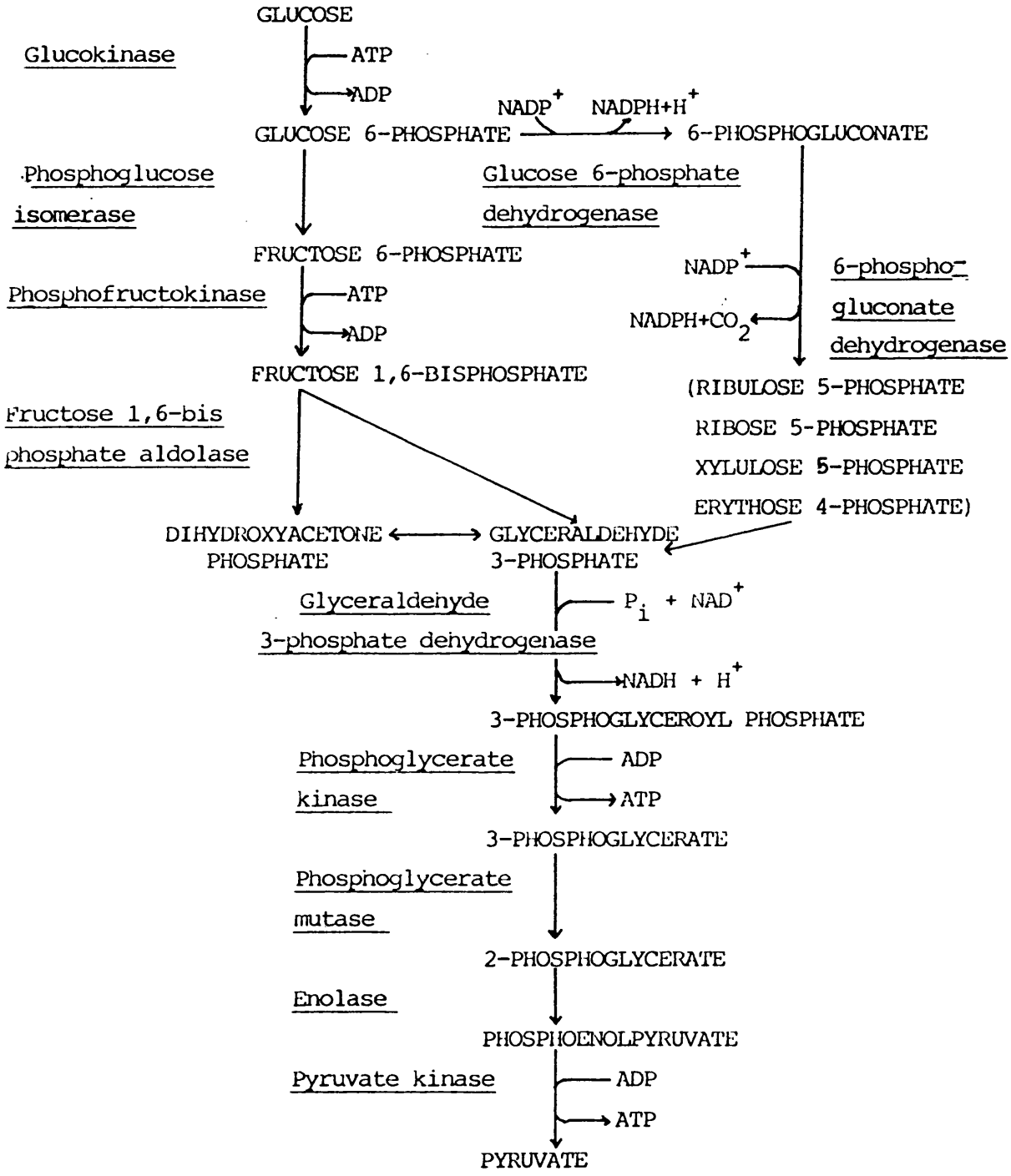
Having introduced the archaebacteria and the organisms studied, it is now important to discuss the pathways of glucose catabolism in each of the kingdoms, the eubacteria the eukaryotes, and the archaebacteria. This discussion highlights the absence of information about the existence of such pathways in the archaebacteria.

The universal occurrence of sugars and their key role in biosynthesis has lead to the suggestion that a sugar based biochemistry was an early evolutionary innovation (Gest & Schopf, 1983 . It is this possibility that led to the present investigation of glucose catabolism in the archaebacteria.

1.3.1. Catabolism in eubacteria and eukaryotes.

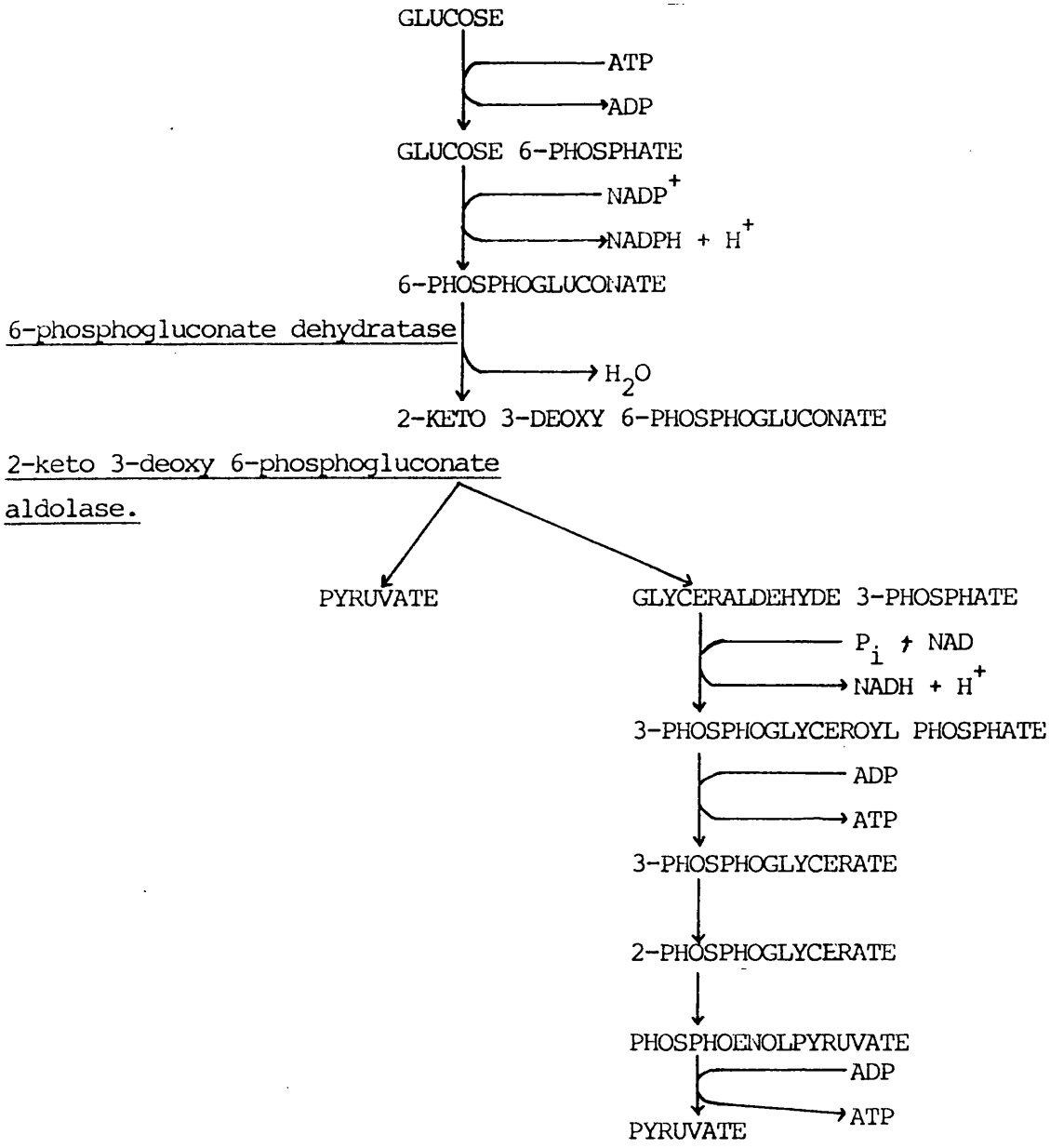
Payton & Haddock (1985), and Cooper (1986) have comprehensively reviewed the catabolic pathways leading to pyruvate from glucose. The Embden-Meyerhof pathway (figure 1.3. is characteristic of eukaryotes, and a number of anaerobic and facultative anaerobic eubacteria. A key enzyme in this pathway is 6-phosphofructokinase which catalyses the ATP-dependant conversion of fructose 6-phosphate to fructose 1,6-bisphosphate however this activity is absent from many strictly aerobic eubacteria, which suggests this pathway is inoperative in these organisms. In such eubacteria glucose is catabolised via the Entner Doudoroff pathway (Entner & Doudoroff, 1952) which depends on the conversion of 2-keto 3-deoxy 6-phosphogluconate

Figure 1.3. The Embden-Meyerhof and Pentose phosphate Pathway.



The Embden-Meyerhof pathway and pentose phosphate pathway are present in both eubacteria and eukaryotes.The sequence of reactions from glyceraldehyde 3-phosphate to pyruvate are common to the conventional and modified Entner-Doudoroff pathways.

Figure 1.4 The Conventional Entner-Doudoroff Pathway.



The conventional Entner-Doudoroff pathway is found in a number of eubacteria, especially psueudomonads.

into glyceraldehyde 3-phosphate and pyruvate (Figure 1.4). The glyceraldehyde 3-phosphate is metabolised to give a further molecule of pyruvate via a sequence common to the Embden-Meyerof pathway.

Another pathway of glucose catabolism found in eukaryotes and eubacteria is the hexose-monophosphate pathway (Racker 1948,1957). Rather than the oxidation of glucose, the pathway probably serves to provide NADPH, pentose and tetrose sugars.

Some species of eubacteria possess the hexose phosphates as found in the pentose-phosphate phosphoketolase pathway (Heath et al., 1956) and the hexose phosphate-pentose phosphate phosphoketolase pathway (Scardovi & Trovatelli, 1965; De Vries et al., 1967).

Many Enterobacteriaceae and some clostridia and pseudomonads possess the methyl glyoxal pathway (Cooper, 1984, 1986). Dihydroxyacetone phosphate is converted to methyl glyoxyal and then to pyruvate without the generation of ATP.

Catabolism of glucose by eubacteria and eukaryotes has a common sequence of reactions converting glyceraldehyde 3-phosphate to pyruvate which yields two molecules of ATP from ADP and Pi. It is of interest to show if the archaebacteria also possessed this highly conserved set of reactions.

1.3.2. Catabolism of glucose by the archaebacteria.

In this section the catabolism of glucose in each group of the archaebacteria, the extreme halophiles, the

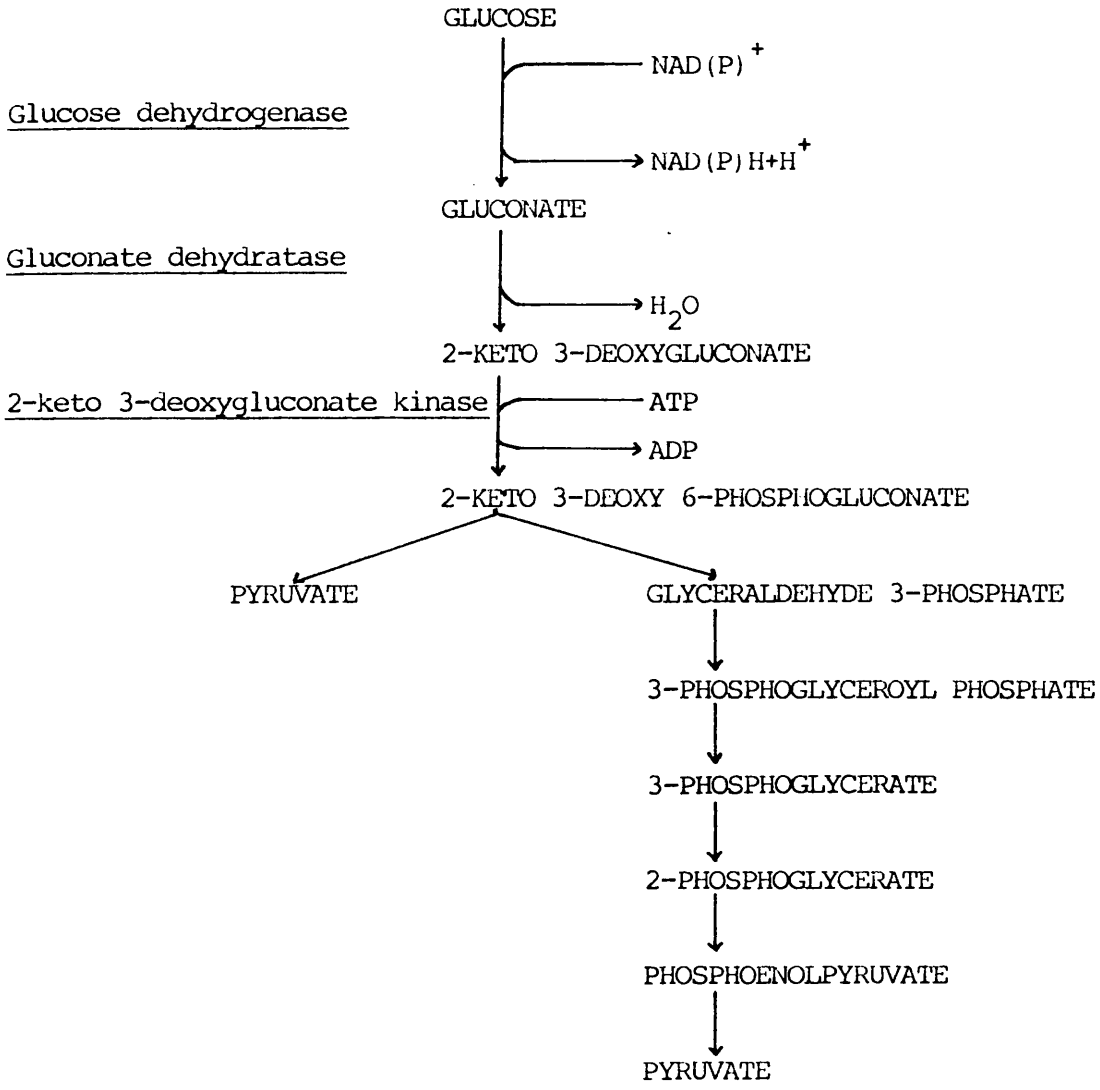
thermophiles and the methanogens , is reviewed.

1.3.2.1. Halophiles.

Many extreme halophiles use proteins and amino acids, rather than carbohydrates, as their sole carbon source for growth (Larsen, 1981). However, strains of Halobacterium saccharovorum isolated by Tomlinson & Hochstein (1972) were shown to utilize carbohydrates. One strain of H. saccharovorum has been shown to catabolise glucose and galactose via a modified Entner-Doudoroff pathway(Figure 1.5.) (Tomlinson et al., 1974). Glucose is initially oxidised to gluconate by an NAD-dependant glucose dehydrogenase and this is then dehydrated to 2-keto 3-deoxygluconate. Subsequent phosphorylation with ATP forming 2-keto 3-deoxy-6-phosphogluconate which undergoes aldol-cleavage to equimolar amounts of pyruvate and glyceraldehyde 3-phosphate. This pathway differs from the classical Entner-Doudoroff pathway since the phosphorylation only occurs just before the aldol-cleavage. However, Tomlinson et al. (1974) noted that this had no absolute requirement for ATP, for the production of pyruvate. This finding may be important and will be considered further when glucose catabolism in Sulfolobus is discussed.

Tomlinson & Hochstein (1976) named this pathway the modified Entner-Doudoroff pathway. All the enzymes that convert glyceraldehyde 3-phosphate to pyruvate described in the Embden-Meyerhoff pathway were also detected in H. saccharovorum, however the oxidation of 6-phosphogluconate or of glucose 6-phosphate was not observed, suggesting the absence of the conventional Entner-Doudoroff pathway.

Figure 1.5 The modified Entner-Doudoroff Pathway.



The modified Entner-Doudoroff pathway was found in the halophilic archaebacterium H. saccharovorum (Tomlinson et al.,1974), but is also described in a number of eubacteria. The key enzyme activity of the pathway is 2-keto 3-deoxygluconate kinase.

The pathway of glucose catabolism in Halobacterium halobium was studied D'Souza & Altelkar (1983). They were unable to assay fructose 1,6-bisphosphate aldolase activity. Moreover, they were unable to show triose-phosphate formation from glucose, fructose or glucose 6-phosphate. These observations suggest that the Embden-Meyerhof pathway is absent but do not discount either a gluconeogenic pathway or the Entner-Doudoroff pathway.

The modified Entner-Doudoroff pathway of H.saccharovorum is unusual but not unique since it has been reported in Clostridium (Andreesen & Goltschalk, 1969; Bender et al., 1971) Alcaligenes, Achromobacter (Kersters & De Ley a) 1968) and Rhodopseudomonas spheroides (Szymona & Doudoroff, 1960).

1.3.2.2. Thermophiles

In this section on thermophilic archaebacterial metabolism the two groups, thermoacidophiles and thermoproteales, will be dealt with individually.

Thermoacidophiles.

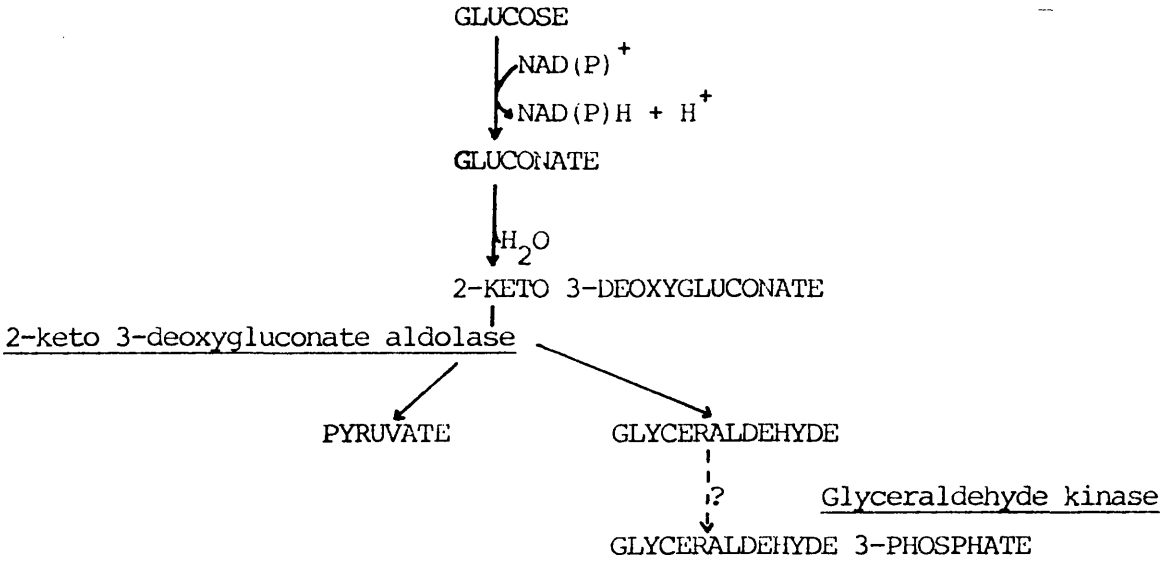
This group is composed of two orders Sulfolobales and Thermoplasmales. Sulfolobus solfataricus was shown by DeRosa et al. (1984) to be capable of growth on glucose as the sole carbon source. The absence of 6-phosphofructokinase activity from S. solfataricus extracts and preliminary investigations with differently labelled ^{14}C-glucoses (DeRosa et al., 1983) indicated that glucose was not catabolised via the glycolytic pathway. However, DeRosa et al.(1984) demonstrated a glucose dehydrogenase activity

converting glucose to gluconate followed by a dehydration to 2-keto 3-deoxygluconate . Upto this point, the reaction scheme is the same as the modified Entner-Doudoroff pathway found in H. saccharovorum, but the next reaction is different. The 2-keto 3-deoxygluconate, rather then being phosphorylated is cleaved by the activity of the hitherto unknown enzyme, 2-keto 3-deoxygluconate aldolase, to glyceraldehyde and pyruvate. This non phosphorylated Entner-Doudoroff pathway (figure 1.6) was deduced from analysis of intermediates and by enzymatic activities. The fate of glyceraldehyde was studied by considering the phosphorylation to glyceraldehyde 3-phosphate by ATP by following ADP formation. Such an activity was shown in cell extracts, although the production of glyceraldehyde 3-phosphate could not be shown.

Using a radiorespirometeric assay Wood et al.(1987) have provided evidence for the in vivo operation of this pathway in Sulfolobus brierleyi and Sulfolobus strian LM (similar to Sulfolobus acidocaldaricus). The release of $^{14}CO_2$ from glucose specifically labelled in carbon atoms 1,2,3 and 4, and 6 was measured in growing cultures of the organisms. Substantial release of carbon atoms 1 and 4 by both strains was consistent with a modified, non phosphorylated, or conventional Entner-Doudoroff pathway. The release of more C1 than C4 suggested that an oxidative pentose phosphate pathway may also be operative although the low release of C2 in S. brierleyi indicates that the pathway is not cyclic.

Glucose catabolism by Thermoplasma acidophilum, the

Figure 1.6. The Non-Phosphorylated Entner-Doudoroff Pathway.



The non-phosphorylated Entner-Doudoroff pathway involves no phosphorylated hexoses, and has the key enzyme activity of 2-keto 3-deoxygluconate aldolase. It has only so far been described in the archaebacterium Sulfolobus solfataricus (De Rosa et al., 1984). The formation of glyceraldehyde 3-phosphate was not identified.

sole representative of the Thermoplasmales, was far from clear before the commencement of the work reported in this thesis. No enzymatic activities associated with glucose catabolism had been identified. Only Searcy & Whatley (1984) had studied the metabolism in Tp. acidophilum. From measurements of O_2 consumption by extracts with various substrates, they concluded that the Embden-Meyerhof and the pentose phosphate pathways were active. Enzymatic analysis showed that the conventional Entner-Doudoroff pathway was not active; however, growth on ^{14}C-[U]glucose showed that glucolactone was an intermediate of catabolism but no phosphorylated hexoses were detected.

This contradictory evidence and the absence of a complete survey of key enzymes in Tp. acidophilum provided an excellent forum for research.

Thermoproteales.

These organisms are characterised as rigid rods,that optimally grow between 70°C and 96°C and between pH 4.0 and pH 7.0. Members are both heterotrophic and autotrophic.

Carbon sources of Thermoproteus tenax include glucose, sucrose and CO_2. Thermfilum penderis, Thermococcus celer and Thermodiscus marittimus can all grow heterotrophically on yeast extract and bacto-tryptone proteins. However, Desulfurococcus saccharvorans can metabolise glycogen and glucose (Stetter & Zillig 1985). The extremely thermophilic Pyrodicium occultum is autotrophic strictly requiring hydrogen and sulphur, although growth is stimulated by yeast extract.

Although growth studies on these organisms do indicate that some of them are capable of metabolising glucose, no pathways or relevant enzymatic activities have been described.

1.3.2.3.. The methanogens.

Methanogenic archaebacteria are all autotrophs, obtaining energy from the reduction of CO_2 by H_2 to form CH_4. The assimilation of carbon by the methanogens has been extensively reviewed by Fuchs & Stupperich (1984,1986), Kirsop (1984), Whitman (1985), Zeikus et al.(1985) and Jones et al.(1987).

Using ^{13}C-NMR spectroscopy Evans et al.(1985,1986) demonstrated the pathways of carbohydrate turnover in Methanobacterium thermoautotrophicum. Although this organism does not require glucose for its energy or as carbon source, it did catabolise it. [1]-^{13}C-glucose and [6]-^{13}C glucose fed to growing cells led to the formation of triose phosphates that were only labelled in the C-3 position, a labelling pattern suggestive of the Embden-Meyerhof pathway. Enzymatic analysis described by Fuchs et al. (1983) showed M. thermoautotrophicum to possess a unidirectional fructose 1,6-bisphosphate aldolase, that is only detectable in the direction of aldol condensation and thus providing a route for glucose synthesis. No phosphofructokinase activity was detected, supporting the unidirectional pathway. However it does not explain the route of glucose catabolism, which it should possess to enable it to breakdown hexoses formed. in the light of the pathways found in some halobacteria and thermoacidophiles it is of interest to show the pathway of glucose catabolism in the methanogens.

1.4. Concluding remarks

Although the survey of glucose catabolism in the archaebacteria is far from complete, it is worth pointing out that no 6-phosphofructokinase activity has been reported in archaebacterial species. It is also interesting to note that some members possess unique variations of the Entner-Doudoroff pathway. Consideration of these findings coupled with tneir evolutionary implications promised that the investigation of the catabolism of glucose by Thermoplasma acidophilum would be intriguing and informative.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 MATERIALS.

2.1.1 Organisms

Viable cultures of both Thermoplasma acidophilum (D.S.M. strain 1728) and Sulfolobus acidocaldarius (D.S.M. strain 639) were obtained from the Deutsche Sammlung von Mikroorganismen Culture Collection, Gottingen, W. Germany, approximately every 6 months.

2.1.2. Chemicals.

All chemicals used in the enzyme assays were of high purity.Cofactors such as $NADP^+$,NAD^+, ATP and ADP were obtained from Boehringer, Mannheim, W.Germany. Substrates were obtained from Sigma Chemical Company,Poole,Dorset,U.K.

Yeast extract used in the growth media was supplied by Difco Laboratories,Detroit,Michigan, U.S.A.

D-[1-^{14}C] and D-[2-^{14}C] glucose were obtained from Amersham International,Amersham,U.K. and D-[3,4-^{14}C]glucose was supplied by New England Nuclear, Boston,U.S.A.

2.1.3 Purified enzymes.

All purified enzymes used in coupled assays were obtained from Boehringer, Mannheim, W.Germany except for phosphoacyltransferase,yeast enolase rabbit muscle fructose 1, 6-bis phosphatase which were ordered from Sigma Chemical Company, Poole, U.K. Poole,U.K.

2.1.4 Chromatography materials

Precoated silica gel thin-layer chromatography plates were supplied by Merck,Poole,Dorset and the chromatography paper was obtained from Whatman Lab.Sales, Maidstone, Kent.

2.2 METHODS.

2.2.1 Growth and harvest of Thermoplasma acidophilum

Thermoplasma acidophilum was grown in medium 158 described in the D.S.M. catalogue (Darland et al., 1970). It contained the following compounds in a litre:1.32g NH_4SO_4,0.37g KH_2PO_4,0.25g $MgSO_4.7H_2O$,0.056g $CaCl_2$,1.93mg $ZnSO_4.H_2O$,0.05mg $CuCl_2.2H_2O$,0.02mg $NaMoO_4$,0.03mg $VOSO_4$, 0.019mg $CoSO_4.7H_2O$,1.0g yeast extract and 10g of D-glucose. The pH was adjusted at room temperature to pH 2.0 with 5M H_2SO_4. 1 litre of sterile medium was usually inoculated with 0.1%(v/v) of liquid culture of Tp. acidophilum with a A_{650} of 0.2-0.3 and incubated at 60°C in a shaking incubator (50-150 rev/min) in a 2.5 litre conical flask.

After 3-4 days, when an absorbance of approximately 0.30 units at 650nm was reached,the cells were harvested. The culture was centrifuged at 3300g for 15min at room temperature and the pelleted cells were collected.

2.2.2 Growth and harvest of Sulfolobus acidocaldarius.

Sulfolobus acidocaldarius was grown in medium 639 described in the D.S.M. catalogue of strains (1983).

It had the same mineral salt and yeast extract concentrations as the Thermoplasma acidophilum medium but with the omission of glucose. The pH was adjusted at room temperature to pH 2.0 with 5M H_2SO_4. Using a 10-20%(v/v) inoculum of a stationary phase liquid culture of Sulfolobus acidocaldarius, the cells were grown at 70°C in a shaking incubator (150 rev/min).

The cells were harvested at an absorbance of 0.40 measured at 650nm; this was after 6 days if grown at 60°C or 3 days if grown at 70°C. The culture was centrifuged at 3300g for 15 min. at room temperature and the pellet collected.

2.2.3 Maintenance of cultures.

Since no solid culture medium was available for either of the thermoacidophiles, the cells had to be maintained in liquid culture; however, because the viability of the organisms is lost within a week at room temperature in liquid cultures, the cells were grown in discontinuous culture.

Storage of both Tp. acidophilum and S. acidocaldarius was achieved for 1 month by neutralising the culture with sterile $CaCO_3$ (4g/l). Liquid nitrogen was used for longer term storage, but it proved unreliable. Sterile $CaCO_3$ was added in excess(4g/l) to a static culture and the precipitate was allowed to settle out. 10ml of the supernatant was centrifuged to pellet the cells, which were then resuspended in 1ml of fresh neutralised medium containing 10%(v/v) glycerol. The ampoules were first cooled down to -80°C at a rate

of 1°C per min, then placed in the vapour phase of a liquid nitrogen container for up to 6 months. Cultures were regrown in 100ml of fresh media inoculated with 1ml of cell suspension.

2.2.4 Cell extraction

Cells of either Tp. acidophilum or S. acidocaldarius (0.2-0.3g wet weight) were resuspended in 1ml of 100mM Tris/HCl buffer, pH8.0, and were sonicated at 0°C for three periods of 30s at 40W with a 3mm probe on an ultrasonic disintegrator. The cell-debris was removed by centrifugation at 10,000g and the supernatant was dialysed against 100mM Tris/HCl pH8.0.

2.2.5 Enzyme assays

All enzymes from the thermoacidophiles were assayed at 55°C in a temperature calibrated spectrophotometer and the mesophilic, commercially-prepared enzymes were assayed at 40°C in a 1cm pathlength cuvette in a total volume of 1ml, unless otherwise stated. All buffers were pH corrected at room temperature.

1. Acetate kinase (EC 2.7.2.1)

$$\text{Acetate} + \text{ATP} \longrightarrow \text{Acetyl-phosphate} + \text{ADP}$$

Acetate kinase was assayed in a coupled assay containing 0.1M triethanolamine, pH7.5, 10mM $MgCl_2$ 10mM KCl, 10mM ATP, 0.2mM NADH, 1.0mM phosphoenolpyruvate, 7.5 units of pig heart lactate dehydrogenase (LDH), 12.5 units of rabbit muscle pyruvate kinase and 0.2M sodium acetate. The oxidation of NADH was followed at 340nm after the addition of acetate (Suzuki et al., 1982).

2. Acetyl-CoA synthetase (AMP dependant EC 6.2.1.1) and acetyl-CoA synthetase (ADP dependant EC 6.2.1.13)

Acetate+CoA+ATP $\longleftrightarrow$ Acetyl-CoA+AMP+PPi

Acetate+CoA+ATP $\longleftrightarrow$ Acetyl-CoA+ADP+P_i

Both acetyl-CoA synthetases were assayed under the the following conditions.

Firstly,acetyl-CoA synthetase was assayed in 100mM Tris/HCl buffer,pH8.0, 0.30mM CoA, 10mM sodium acetate, 0.10mM ATP, 10mM $MgSO_4$, 0.14 mM 2-mercaptoethanol and 10mM NH_4SO_4. The formation of the thioester bond in acetyl-CoA was monitored at 233nm(Bergmeyer et al., 1963).

The formation of CoA was monitored using 5,5' dithiobis(2-nitrobenzoic acid) (DTNB) and the change in A_{412} was followed in 100mM Tris/HCl, pH 8.0, 10mM $MgSO_4$, 10mM $(NH_4)_2SO_4$, 0.1mM acetyl CoA, 0.25mM ADP 0.12mM DTNB and 0.10mM potassium dihydrogen phosphate. This assay mixture was used to detect the ADP-dependant enzyme;however, for the AMP-dependant acetyl-CoA synthetase was assayed with 0.25mM AMP and 0.10mM potassium pyrophosphate in the place of ADP and potassium dihydrogen phosphate in the above assay.

Acetyl-CoA synthetase [EC 6.2.1.13] only was assayed following the production of ADP from ATP in a coupled assay containing 100mM Tris/HCl, pH7.4, 10mM 2-mercaptoethanol, 0.14mM CoA, 10mM phosphoenolpyruvate, 1.5 units of pig heart LDH, 2.5 units of rabbit muscle pyruvate kinase,10mM sodium acetate and 0.25mM ATP. The oxidation

of NADH was followed at 340nm after the addition of either acetate or CoA (Suzuki et al.,1982).

3. Enolase [EC 4.2.1.11].

D 2-Phosphoglycerate $\longleftrightarrow$ phosphoenolpyruvate+H_2O

Enolase activity was assayed in 100mM Tris/HCl pH 8.0 10mM $MgCl_2$, D 2-phosphoglycerate. The reaction was started with enzyme and its progress was monitored by the increase in A_{240} as described by Westhead (1966).

4. Fructose 1 6-bisphosphatase [EC 3.1.3.11].

Fructose 1,6-bisphosphate $\longrightarrow$ Fructose 6-phosphate+P_i

Fructose 1,6-bisphosphatase was assayed at 40°C by following the increase in A_{340} in a coupled assay containing 50mM Tris/HCl, pH8.0, 0.4mM $NADP^+$, 1mM fructose 1,6-bisphosphate (FDP), 1.5 units of rabbit muscle glucose 6-phosphate dehydrogenase, 3.0 units of yeast phosphoglucose isomerase, 10mM $MgCl_2$, 20mM $MnCl_2$ and 14mM 2-mercaptoethanol (Fuchs et al.,1983).

5. Fructose 1,6-bisphosphate aldolase [EC 4.1.2.13]

Fructose 1,6-P $\longleftrightarrow$ Dihydroxyacetone-P+Glyceraldehyde,3-P

Fructose 1,6-bisphosphate aldolase was assayed by monitoring either the production of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate or fructose 1,6-bisphosphate. The former were assayed at 40°C in 100mM Tris/HCl, pH7.4, 2.0mM NAD^+, 8.5mM sodium arsenate, 0.8 units of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, 14.0mM 2-mercaptoethanol and 1.7mM fructose 1,6 bisphosphate. The reaction was started with the addition of enzyme and its progress was monitored by the increase in A_{340} (Taylor 1955).

6.Fructose 1-phosphate aldolase [EC 4.1.2.13].

Fructose 1-phosphate ⟶ glyceraldehyde+dihydroxyacetone phosphate

Fructose 1-phosphate aldolase was assayed at 40°C in 100mM Tris/HCl, pH8.0, 0.20mM NADH, 0.4 units of rabbit muscle sn-glcerol 3-phosphate dehydrogenase and 4.0mM fructose 1-phosphate. NADH oxidase was assayed prior to the addition of fructose 1-phosphate by following the decrease in A_{340}. This method was adapted from Ling et al. (1955).

7.Gluconate dehydratase [EC 4.2.1.39].

Gluconate ⟶ 2-keto 3-deoxygluconate $+H_2O$

Gluconate dehydratase activity was assayed in 100mM Tris/HCl, pH8.0, 10mM $MgCl_2$ and 50mM potassium gluconate. The production of 2-keto 3-deoxygluconate was determined discontinuously by the periodate-thiobarbituric assay of Weissbach & Huritz (1959). The timed incubation was stopped with 1%(w/v) trichloroacetic acid and the mixture was centrifuged for 5 min at 10,000g. A 0.2ml aliquot was taken and incubated for 30min at 37°C with 125 µl of 25mM $NaIO_4$ in 0.125M H_2SO_4. To this 0.1ml of 2%(w/v) $NaAsO_4$ in 0.5M HCl was added to stop the oxidation, then 1.0ml of 0.3%(w/v) 2-thiobarbituric acid, pH2.0, was added and the absorbance at 549nm was measured (E^{549}= $67.8x10^3$ $1mol^{-1}cm^{-1}$) (Kersters & De Ley, b) 1968).

8. Gluconate dehydratase (EC 4.2.1.39) and 2-keto 3-deoxygluconate aldolase.

Gluconate ⟶ 2-keto 3-deoxygluconate ⟶ glyceraldehyde + pyruvate

The combined activities of D-gluconate dehydratase and 2-keto 3-deoxygluconate aldolase were assayed discontinuously in 100mM Tris/HCl, pH8.0, 10mM D-gluconate and 10mM $MgCl_2$. After cooling the assay to 4°C, pyruvate formation was determined spectrophotometrically at 340nm with 0.01 units of pig heart LDH and 0.10mM NADH. This method was adapted from Czok & Lamprecht (1974).

9.Glucose dehydrogenase [EC 1.1.1.47].

Glucose + $NADP^+$ ⟶ gluconate + NADPH + H^+

Glucose dehydrogenase activity was assayed in 100mM Tris/HCl, pH8.0, 0.4mM $NADP^+$, 1.0mM glucose 6-phosphate. The reaction was started with enzyme and its progress monitored by the increase in A_{340} (De-Moss, 1955).

11. Glyceraldehyde dehydrogenase [EC 1.2.1.5.].

Glyceraldehyde + $NADP^+$ ⟶ glycerate + NADPH+H^+

Glyceraldehyde dehydrogenase activity was measured by monitoring the reduction of $NADP^+$ at 340nm in a reaction mixture containig 100mM Tris/HCl, pH8.0, 0.20mM $NADP^+$ and dedimerised 5.0mM DL-glyceraldehyde (Westernausen et al.,1982).

12.Glyceraldehyde kinase (triokinase EC 2.7.1.28).

Glyceraldehyde + ATP ⟶ glyceraldehyde 3-phosphate + ADP

Glyceraldehyde kinase was assayed at 40°C in a coupled assay which detected the production of glyceraldehyde 3-phosphate. Assay mixtures contained 100mM Tris/HCl, pH8.0, 10mM $MgCl_2$, 10mM KCl, 0.2 units of yeast triose phosphate isomerase, 1.0mM ATP, 0.2mM NADH and 5.0mM DL-glyceraldehyde. The reaction was started with DL-glyceraldehyde after prior determination of the NADH oxidase activity; the decrease in A_{340} was followed (Hers,1961).

The formation of ADP from ATP was also measured in a coupled assay containing 100mM Tris/HCl, pH8.0, 0.2mM NADH, 10mM phosphoènolpyruvate, 10mM $MgCl_2$, 10mM KCl, 12.5 units of rabbit muscle pyruvate kinase 7.5 units of pig heart LDH and 10mM DL-glyceraldehyde. The A_{340} was followed after the rates were corrected for ATPase and NADH oxidase activities.

13.Glyceraldehyde 3-phosphate dehydrogenase [EC 1.2.1.12]

glyceraldehyde 3-phosphate + NAD^+ + P_i ⟶ 1-glyceryl 3-phosphate + $NADH+H^+$

Glyceraldehyde 3- phosphate dehydrogenase was assayed in 100mM Tris/HCl, pH8.0, containing 20mM sodium arsenate, 3.3mM DL glyceraldehyde 3-phosphate with either 1.0mM NAD^+ or 0.4mM $NADP^+$. The reaction was started with enzyme and the increase in A_{340} was followed with time (Krebs, 1955).

14. Glyceraldehyde reductase (glycerol dehydrogenase EC 1.1.1.72).

Glyceraldehyde + NADPH + $H^+ \longleftrightarrow$ glycerol + $NADP^+$

Glyceraldehyde reductase was assayed by monitoring either the production of glycerol or, in the reverse direction, the production of glyceraldehyde. The reaction in the direction of glycerol was in 0.1M triethanolamine buffer, pH 7.0,10mM $MgCl_2$, 14mM 2-mercaptoethanol, 0.2mM NADPH and 50mM dedimerised DL-glyceraldehyde. NADPH oxidase activity was measured prior to the addition of glyceraldehyde and the A_{340} was followed with time (Flynn et al.,1982).

The oxidation of glycerol was assayed from the increase in A_{340} in 0.2M diethanolamine, pH 8.7, 1.0M glycerol, 14mM 2-mercaptoethanol, 10mM $MgCl_2$ and 5.0mM $NADP^+$ (Flynn et al., 1982).

15. Glycerate kinase [EC 2.7.1.31].

glycerate + ATP $\longrightarrow$ 2-phosphoglycerate + ADP

Glycerate kinase activity was assayed at 40°C in a coupled assay containing 100mM Tris/HCl, pH 8.0, 10mM $MgCl_2$, 10mM KCl, 0.3 units of yeast enolase, 25 units of rabbit muscle pyruvate kinase, 1.5 unit of pig heart LDH, 0.2mM NADH, 5mM ATP, 1.0mM ADP, 5mM DL-calcium glycerate and 5mM EGTA. The reaction was started with calcium glycerate after monitoring the NADH oxidase by following the decrease in A_{340}.

16. Glycerol dehydrogenase [EC 1.1.1.6].

Glycerol + NAD(P) + H^+ $\longleftrightarrow$ dihydroxyacetone + NAD(P)H

Glycerol dehydrogenase was assayed in 0.3M triethanolamine, pH 7.3, 10mM $MgCl_2$, 14mM 2-mercaptoethanol, 10mM dihydroxyacetone and 0.2 mM NAD(P)H. The decrease in A_{340} was followed with either cofactor after the addition of dihydroxyacetone (Burton, 1955).

17. Glycerol kinase [EC 2.7.1.30].

Glycerol + ATP $\longrightarrow$ DL-glycerolphosphate + ADP

Glycerol kinase activity was assayed with a coupled assay at 40°C in 100mM Tris/HCl, pH 8.0, 0.2 mM NADH, 1.0mM phosphoenolpyruvate, 10mM $MgCl_2$, 10mM KCl, 12.5 units of rabbit muscle pyruvate kinase, 7.5 units of pig heart LDH and 100mM glycerol. The decrease in A_{340} was followed after addition of glycerol to correct for ATPase activity (Chernick, 1978).

18. (sn)-Glycerol 3-phosphate dehydrogenase ($NAD^{\pm}$ dependant [EC 1.1.1.8] and $NADP^{\pm}$ [EC 1.1.1.94].

Dihydroxyacetone + NAD(p)H + H^+ $\longleftrightarrow$ sn-glycerol 3-phosphate + $NAD(P)^+$

sn-Glycerol 3-phosphate dehydrogenase was assayed in 0.3M triethanolamine, pH 7.3, 0.2mM NAD(P)H and 0.5mM dihydroxyacetone phosphate. The decrease in A_{340} was measured with both cofactors after the addition of dihydroxyacetone phosphate (Beisenherz et al., 1955).

The formation of dihydroxyacetone phosphate from DL-glycerol phosphate was assayed in 0.2M diethanolamine,

pH 9.3, containing 1.6mM $NAD(P)^+$ and either 25mM DL-glycerol 3-phosphate or (sn)-glycerol 3-phosphate. The increase in A_{340} was followed with time (Nielsel et al., 1982).

19. 6-Phosphofructokinase [EC 2.7.1.11].

Fructose 6-phosphate + ATP $\longrightarrow$ fructose 1 6-bisphosphate + ADP

Phosphofructokinase activity was assayed at 40°C in a coupled assay containing 100mM Tris/HCl, pH 8.0, 10mM NH_4Cl, 10mM $MgCl_2$, 0.2mM NADH, 1.0mM fructose 6-phosphate, 0.12-units of rabbit muscle fructose 1,6-bisphosphate aldolase 6.0 units of yeast triose phosphate isomerase and 1.7 units of rabbit muscle (sn)-glycerol 3-phosphate dehydrogenase.

The reaction was started with fructose 6- phosphate and its progress monitored by the decrease in A_{340} after correction for the NADH oxidase rate (Ling et al.,1955).

20. Phosphoglycerate mutase [EC 5.4.2.1].

3-phosphoglycerate $\longleftrightarrow$ 2-phosphoglycerate

Phosphoglycerate mutase activity was assayed by following the increase in A_{240} at 40°C due to the formation of phosphoenolpyruvate in 100mM Tris/HCl, pH 8.0, 10mM D 3-phosphoglycerate, 10mM $MgCl_2$, 3.0 units of yeast enolase, 50mM KCl and 1.0mM 2,3-bis phospho-glyceric acid (Westhead, 1966).

21. Phosphotransacetylase [EC 2.3.1.8].

Acetyl-phosphate + CoA $\longleftrightarrow$ acetyl-CoA + P_i

Phosphotransacetylase was assayed in 100mM Tris/HCl, pH 7.5, 10mM $(NH_4)_2SO_4$, 10mM $MgCl_2$, 0.12mM DTNB, 0.10mM acetyl-CoA and 10mM potassium dihydrogen phosphate and the increase in A_{412} was followed after phosphate was added.

Phosphotransacetylase activity was also assayed by following the formation of acetyl-CoA in 100mM Tris/Hcl, pH 7.5, 10mM $(NH_4)_2SO_4$, 8.0mM acetyl-phosphate and 0.3mM CoA. The formation of the thioester bond was monitored at 233nm (Bergmeyer et al.,1963).

22. Phosphoglucose isomerase [EC 2.7.5.1].

Fructose 6-phosphate $\longleftrightarrow$ glucose 6-phosphate

Phosphoglucose isomerase was assayed at 40°C in 100mM Tris/HCl, pH 8.0, 1.0mM D-fructose 6-phosphate, 1.5 units of rabbit muscle glucose 6-phosphate dehydrogenase and 0.4mM NADP. The reaction was started with enzyme and its progress monitored by the increase in A_{340} (Slein, 1955).

23. Pyruvate kinase (EC 2.7.1.40).

Phosphoenolpyruvate + ADP $\longrightarrow$ pyruvate + ATP

Pyruvate kinase activity was assayed at 340nm in a coupled assay containing 100mM Tris/HCl, pH 8.0, 10mM $MgCl_2$,10mM KCl 5.0mM ADP, 0.20mM NADH, 1.5 units of pig heart lactate dehydrogenase and 10.0mM phosphoenolpyruvate.. The. reaction was started bythe addition of phosphoenolpyruvate after first assessing the NADH oxidase activity (Valetine & Tanaka, 1966).

24, Triose-phosphate isomerase [EC 5.3.1.1].

Dihydroxyacetone-P<————————>glyceraldehyde 3-P

Triose phosphate isomerase activity was assayed at 40°C in 100mM Tris/HCl, pH 8.0, containing 0.2mM NADH, 2.6mM DL-glyceraldehyde 3-phosphate and 0.4 units of rabbit muscle (sn)-glycerol 3-phosphate dehydrogenase. The reaction was started with glyceraldehyde 3-phosphate after the NADH oxidase rate had been determined and its progress monitored by the decrease in A_{340} (Beiseenherz, 1955).

2.2.6. Preparation of substrates.

1. Preparation of acetyl-Coenzyme A

Acetyl-CoA was prepared by the method of Stadtman (1957). 10mg of CoA were dissolved in 1ml of double distilled water and the solution cooled on ice. 0.2ml of 1.0 M $KHCO_3$ was added to bring the solution to pH7.5. 0.2ml of freshly diluted 0.1M acetic anhydride was added and the solution left on ice for 10min . Acetylation was tested with DTNB at 412nm.

2. Preparation of DL-glyceraldehyde.

A known quantity of DL-glyceraldehyde was dissolved in 1ml of double distilled water and heated at 100°C for 5min to break the dimers formed in solution.

2.2.7 Product Analysis

1. Enzymatic

a) Glucose

Glucose concentrations were estimated in an assay

containing 0.10M triethanolamine, pH 7.5, 10mM KCl, 10mM $MgCl_2$, 1.0mM ATP, 0.6mM $NADP^+$, 3.6 units of rabbit muscle hexokinase [EC 2.7.1.1] and 1.8 units of glucose-6-phosphate dehydrogenase [EC 1.1.1.49]. The increase in A_{340} was measured at 30°C in a 1 cm pathlength cuvette with a total volume of 1ml (Bergmeyer et al., 1974).

b) Acetate.

Acetate concentrations were estimated in the following assay containing 0.1M triethanolamine, pH 7.5, 10mM $MgCl_2$ 10mM KCl, 1.0mM ATP, 0.2mM NADH, 1.0mM phosphoenolpyruvate 12,5 units of pig heart pyruvate kinase, 7.5 units of pig heart LDH and 4.0 units of E. coli acetate kinase. Up to 100µl of the media was incubated at 25°C and the initial increase in A_{340} was monitored and compared with that produced by a set of standard actate concentrations (Suzuki et al., 1982).

c) Pyruvate.

Pyruvate concentrations were estimated at 20°C in the following assay containing 100mM Tris/HCl, pH 8.0 0.10mM NADH, and 0.01 units of pig heart LDH. The increase in A_{340} was monitored after addition of the sample (Czok & Lamprecht, 1974).

d) Glyceraldehyde.

Glyceraldehyde concentrations were estimated in an assay containing 100mM Tris/HCl, pH 8.0, 10mM KCl, 1.0

unit of yeast alcohol dehydrogenase and 0.2mM NADH. The decrease in A_{340} was measured after the addition of the sample (Racker, 1957).

2. Chromatographic.

a) Pyruvate and glyceraldehyde.

Freshly prepared cell extracts were incubated in 100mM Tris/HCl, pH 8.0, with various substrates and cofactors for 1-3 hrs at 55°C. To 1ml of this solution, 0.2ml 0f 2%(w/v) 2,4-dinitrophenylhydrazine in 1.0M HCl was added and the mixture was incubated for 10min at 20°C to allow complete reaction with aldehydes and ketones. The resulting coloured 2,4-dinitrophenylhydrazones were extracted into 0.2ml of ethylacetate, and then chromatographed on Kiesgel 80 thin layer chromatography plates.

b) 2-Keto 3-deoxygluconate.

Dialysed homogenates of Tp. acidophilum were incubated for 60 min at 55°C in 100mM Tris/HCl, pH8.0, containing 50mM D-potassium gluconate and 20mM $MgCl_2$. 15mg of Dowex-50 hydrogen form resin were added to the 1ml reaction mixture and, after 5min, 100µl of the decationised mixture was dried down under vacuum. The residue was redissolved in 10µl of double distilled water and applied to a silica gel thin layer chromatography plate, which was then chromatographed in n-propanol/ ammonia 0.88/ 0.2%(w/v) EDTA (6:3:1 by volume) at room temperature.

After drying, the chromatogram was sprayed with ethanolic 0.10%(w/v) o-phenylenediamine in 10%(w/v) trichloracetic acid and heated for 10min at 105°C. The chromatogram was viewed under ultraviolet light (Kersters & De Ley, b) 1968).

2.2.8Radiorespirometeric analysis.

4ml of growth medium (DSM medium 158), containing 0.05g of D-glucose and 0.5 µCi of either D-[1], D-[2] or D-[3,4] ^{14}C-glucose (specific activity 0.01 µCi/mg), was inoculated with 1ml of late log. phase culture of Thermoplasma acidophilum. The cells were grown aerobically at 60°C in a 50ml sterilin tube which contained a LP4 tube with 0.21ml of 5.0M KOH. The KOH was removed from each treatment at regular intervals, mixed with 40 ml of Octiphase 'safe' and the trapped $^{14}CO_2$ estimated in an LKB rack Beta scintilation counter. Fresh KOH was added to the well to replace that taken. To check that the radioactivity was due to $^{14}CO_2$, HCl and sodium bicarbonate were added to the KOH. The evolution of $^{14}CO_2$ was followed with the growth of Tp. acidophilum on the various radiolabelled glucoses.

2.2.9 Radiolabelling and analysis of acetate produced by glucose catabolism.

Cultures of Tp. acidophilum were grown on D-[1], [2], or [3,4] or [U] ^{14}C-glucose (final specific activity 0.01 µCi/mg) under the conditions in the radiorespirometeric analysis. Cells were removed by centrifugation for 15min at 1800g in a bench centrifuge and an equal

volume of diethyl ether was shaken with the supernatant for 3min to extract the acetate produced in the fermentation. A known volume of the diethyl ether was spotted on to a sheet of Whatman N^{o} 3 chromatography paper and developed in propan 2-ol/0.88 ammonia [8:3 (v/v)] solvent. The acetic acid was identified on the paper with phenol red-borate buffer spray and the R_f was determined. The chromatography paper was cut up into 1cm sections along each lane and left overnight in scintillation vials containing 1ml of double-distilled water. 4ml of octiphase 'safe' was added and the relative amounts of ^{14}C-labelled acetate were determined in a LKB rack Beta scintillation counter. This method is based on that described by Searcy & Whatley (1984).

2.2.10. Preparation of phenol red borate buffer indicator.

Phenol red-borate buffer spray contained 0.05M sodium borate buffer, pH 9.2; 0.2%(w/v) phenol red- in ethanol and alkaline methanol in the proportions 1:2:7. Yellow spots on a purple background were produced by compounds with hydroxyl-groups (Hockenhull, 1953).

2.2.11. Preparation of glucose dehydrogenase.

Cells of Tp. acidophilum were sonicated in 100mM Tris/HCl, pH8.0, and the suspension was centrifuged at 10,000g for 5min to remove cell debris. The following procedures were performed at room temperature. Methanol

was added to the supernatant to give a final concentration of 90%(v/v) methanol and the mixture was centrifuged at 1800g for 15min to collect the precipitate. The pellet was washed twice in 55%(v/v) methanol in 100mM Tris/HCl, pH 8.0, and then was twice resuspended in 100mM Tris/HCl, to which methanol was added to give a 30%(v/v) methanol solution. Each time, the pellet was removed by centrifugation and to the combined supernatants, methanol was added to give a final concentration of 70%(v/v). The precipitate was collected by centrifugation and redissolved in 20mM triethanolamine, pH7.5. This solution was centrifuged at 10,000g for 10min and then applied to the Pharmacia FPLC Mono Q cation exchange column equilibrated with 20mM triethanolamine, pH 7.5. The glucose dehydrogenase was eluted with a 0-0.6M NaCl gradient. Fractions containing glucose dehydrogenase were applied to an FPLC Mono P chromatofocusing column equilibrated with 25mM bis-Tris/HCl, pH7.0, and proteins were eluted with a pH gradient provided by polybuffer 74 from pH7.0-4.0. The fractions containing the enzyme were applied to a Pharmacia Superose-12 column preequilibrated with 50mM sodium phosphate buffer , pH7.2, containing 0.15M NaCl. Fractions containing glucose dehydrogenase activity were collected.

2.2.12. Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Discontinuous SDS-PAGE was performed according to

the method of Laemmli (1970). The resolving gel was composed of 10%(w/v) acrylamide, 0.3%(w/v) bis-acrylamide 0.1M Tris buffer, pH8.8, 0.11%(w/v)SDS, 0.084%(w/v) freshly made ammonium persulphate (initiator) and 0.056%(v/v) N,N,N',N'-tetramethylethylenediamide (TEMED) (catalyst). The acrylamide buffer and ammonium persulphate were mixed and degassed before the addition of the SDS and TEMED. The mixture was poured into 20x20 ATTO gel plates, over-layered with 1% (w/v) SDS and allowed to set. The stacking gel was composed of 3.75%(w/v) acrylamide, 0.1%(w/v) bis-acrylamide, 0.125M Tris/HCl, pH6.6, 0.10%(W/V) SDS, 0.075%(w/v) ammonium persulphate 0.075%(w/v) TEMED. This mixture was degassed as before and layered on top of the resolving gel, overlayered, and set with an inserted comb. Samples of approximately 2.0µg of protein were dried in a vacuum desiccator and redissolved in 20µl of 10mM Tris/pH8.8, 4.0%(w/v) SDS, 20%(v/v) glycerol, 10%(v/v) 2-mercaptoethanol and 0.004%(w/v) bromophenol blue; they were then heated to 100°C for 2min before being applied to the gel in 30mM Tris/HCl, pH8.3, 14%(w/v) glycine, and 1%(w/v) SDS. The electrophoresis was stopped after the dye had traveled the length of the gel and the proteins were visualised using the silver staining proceedure described below.

2.2.13. Silver stain.

The proteins separated by the SDS-PAGE were visualised with silver stain as described by Nielson

and Brown, (1984). The gel was fixed for at least 2hrs in 200ml of 10%(w/v) TCA, then washed three times for 10min, in 10%(v/v) ethanol, 5%(v/V) acetic acid before being incubated at 40°C for 1h with 200ml of 3.4mM potassium dichromate in 3.2mM nitric acid. The gel was then washed twice in 200ml of double distilled water and incubated for a further hour at 40°C in freshly prepared 0.2%(w/v) silver nitrate. The gel was then briefly washed once with double distilled water before incubating twice with 100ml of 3%(w/v) Na_2CO_3 containing $2.0x10^{-4}$%(v/v) formaldehyde at 40°C. When the proteins were stained satisfactorily, the gel was washed for 5min in 5%(v/v) acetic acid to stop the reduction and was stored in double distilled water.

2.2.14 Protein assay.

Protein concentrations were determined in cell extracts and partially purified preparations by the method of Lowry et al.(1951). The protein concentration of highly purified preparations were determined by measuring the A_{260} and A_{280} by the method of Warburg & Christian (1942).

CHAPTER 3.

Catabolism of Glucose to Pyruvate

3.1 Introduction.

The aim of the research described in this chapter was to identify the metabolic pathway(s) by which Thermoplasma acidophilum catabolises glucose to pyruvate and to characterise the component enzymes.

As already discussed, Tp. acidophilum is an obligately aerobic heterotroph requiring both yeast extract and glucose for growth. To show how Tp. acidophilum catabolises glucose, cell extracts were assayed for enzyme activities characteristic of the conventional Entner-Doudoroff (Entner & Doudoroff, 1952) ,Embden-Meyerhof , pentose-phosphate, the modified (Tomlinson & Hochstein, 1976), and the non-phosphorylated Entner-Doudoroff pathways (DeRosa etal.,1984). When activities were recorded,the enzyme was characterised with respect to its Michaelis-Menten constants, specific activity and substrate specificities.

In addition to the enzyme assays, products of the cell extract activity with various substrates and cofactors are shown, demonstrating the intermediates and the activities participitating in the pathway. Also the stoichiometry of the pathway was determined by quantifying the products generated.

It was hoped that by using these approaches the pathway(s) of glucose catabolism could be elucidated.

3.2 Results.

3.2.1.Hexose requirement

Tp. acidophilum was grown in 1%(w/v) yeast extract and basal salts medium as described in the method section, with the addition of the following compounds : 50mM galactose, 100mM glycerol, 50mM gluconate K^+, or 50mM glucose. The growth curves of the organism on each substrate are shown in figure 3.2.1. The doubling times for glucose, galactose, and gluconate K+ were 7.5, 40 and 37h respectively. Growth was not observed with glycerol.

3.2.2 Enzymatic assays.

Dialysed cell extracts of Tp. acidophilum were assayed for the enzymes of glucose catabolism, as described in the method section. The Michaelis-Menten constants (Km) and the specific activities ofeeach of the activities discovered are shown in table 3.2.2. Kinetic constants were calculated from the direct-linear plot (Eisenthal & Cornish-Bowden,1974) and the data are presented in the 'half-reciprocal' plot (figures 3.2.3 to 3.2.14). The following enzyme activities were discovered:

Glucose dehydrogenase [EC 1.1.1.47]

$$\text{D-Glucose} + \text{NAD(P)}^+ \longrightarrow \text{D-gluconate} + \text{NAD(P)H} + \text{H}^+$$

The enzyme showed both NAD^+ and $NADP^+$ dependent catalytic activity, although 30mM NAD^+ was required to give an specific activity equal to that obtained with 0.4mM $NADP^+$.

Galactose dehydrogenase

$$\text{D-Galactose} + \text{NAD(P)}^+ \longrightarrow \text{D galactonate} + \text{NAD(P)H} + \text{H}^+$$

This activity was also dependent on NAD^+ and $NADP^+$. A more comprehensive study of both glucose and galactose

Graph 3.2.1. Showing The Growth Of Thermoplasma acidophilum on Glucose, Galactose, Gluconate and Glycerol.

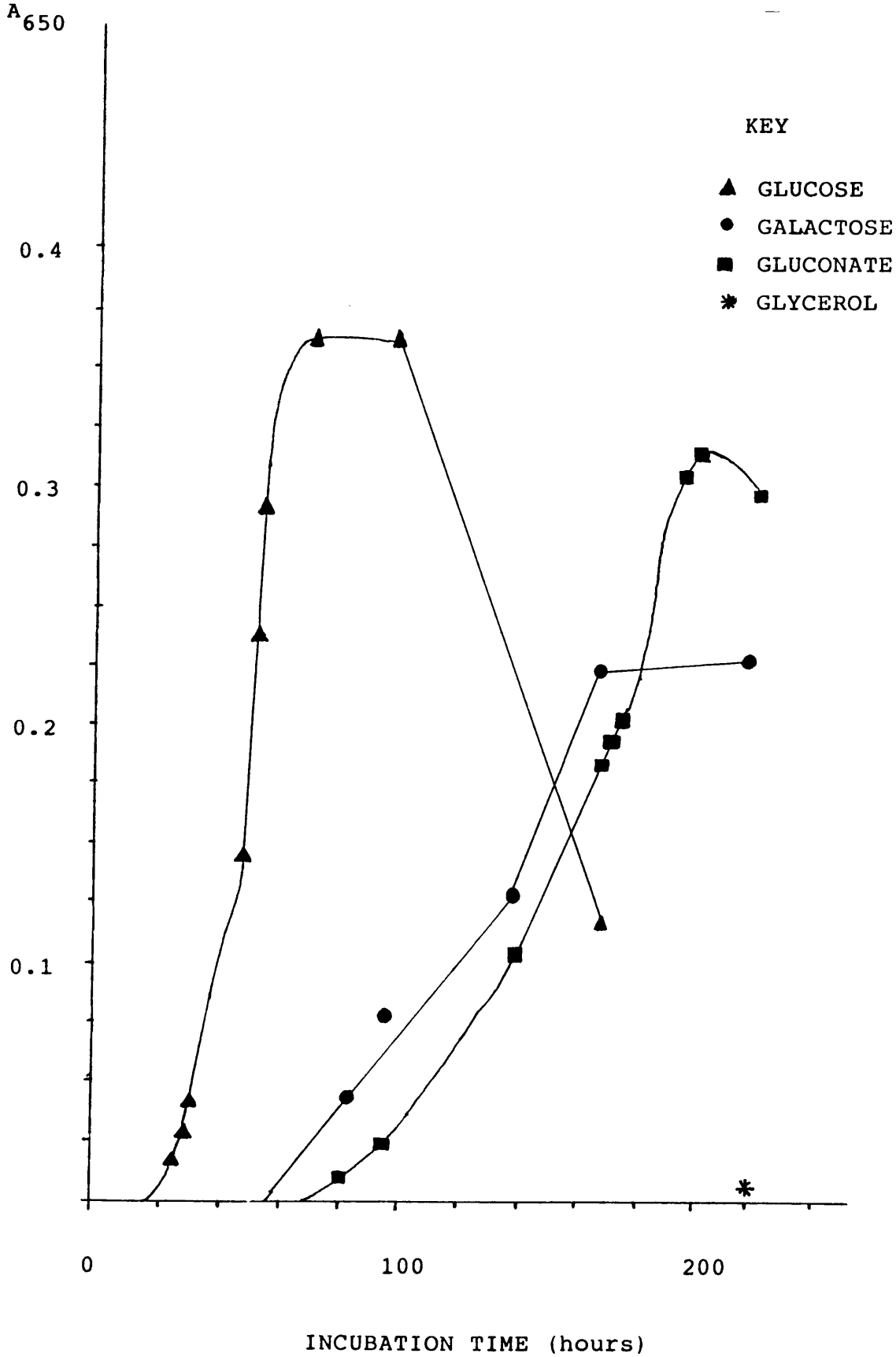


Table 3.2.2. Enzymatic Activities in Cell Extracts of Tp. acidophilum.

Cell extracts of Tp. acidophilum possessed the following enzymatic activities. The specific activities were determined with saturating concentrations and the Km (Michaelis-Menten constants) were calculated from the direct-linear plot (Eisenthal & Cornish-Bowden,1974).

ENZYME	ASSAY TEMP. (°C)	SPECIFIC ACTIVITY ($nmol.min^{-1}mg^{-1}$)	Km VALUES
GLUCOSE DEHYDROGENASE	55	222(±11)	glucose 10.03(±0.85)mM $NADP^+$ 0.045(±0.005)mM NAD^+ >50mM
GALACTOSE DEHYDROGENASE	55	150(±34)	galactose 11.29(±1.06)mM
GLUCONATE DEHYDRATASE	55	3.5(±0.3)	gluconate 5.4(±1.4)mM
GLUCONATE DEHYDRATASE & KDG ALDOLASE	55	4.6(±0.8)	-
GLYCERALDEHYDE DEHYDROGENASE	55	69.6(±5.8)	DL-glyceraldehyde 0.23(±0.013)mM $NADP^+$ 3.7(±0.55)µM
GLYCERATE KINASE	40	29.1(±7.4)	DL-glycerate 0.113(±0.013)mM ATP 1.14(±0.094)mM
ENOLASE	55	141(±15)	2-phosphoglycerate 0.062(±0.0045)mM $MgCl_2$ 0.12(±0.019)mM
PYRUVATE KINASE	55	64.8(±3.3)	ADP 0.102(±0.012)mM phosphoenolpyruvate $S_{0.5}$ 0.50mM
PHOSPHOGLUCOSE ISOMERASE	55	46(±0.5)	fructose 6-phosphate 0.231(±0.0086)mM
TRIOSE PHOSPHATE ISOMERASE	55	310(±50)	
NADH OXIDASE	55	93.5(±18.7)	

Errors are the SEM

dehydrogenase is given in chapter 6.

D-Gluconate dehydratase [EC 4.2.1.39].

D-gluconate ⟶ 2-keto 3-deoxygluconate + H_2O

The enzyme activity was dependant on Mg^{2+}. As shown in the table 3.2.2. the specific activity is very low; optimal conditions may not have been achieved in this particular assay.

D- Gluconate dehydratase and 2-keto 3-deoxygluconate aldolase.

2-Keto 3-deoxygluconate aldolase catalyses the following reaction:

2-Keto 3-deoxygluconate ⟶ D-glyceraldehyde + pyruvate

Both the aldolase and the dehydratase were assayed to together since the substrate 2-keto 3-deoxygluconate was not available. The activities were assayed by monitoring the production of both glyceraldehyde and pyruvate from D-gluconate. The formation of pyruvate form D-gluconate required magnesium ions. The reaction was not dependant on the presence of ATP; however, ATP inhibited the formation of pyruvate, 1mM ATP causing 60% inhibition and 0.5mM ATP causing 50% inhibition of the rate. Both enzymatic activities were unstable in the cell extracts, 50% loss of activity being observed in 24h. The enzyme KDG aldolase is a unique enzyme which is characteristic of the non-phosphorylated Entner-Doudoroff pathway.

DL- Glyceraldehyde dehydrogenase [EC 1.2.1.5].

DL-glyceraldehyde + $NADP^+$ ⟶ DL glycerate + $NADPH+H^+$

This enzyme showed only $NADP^+$ dependency, no activity being detected with NAD^+ in the range of 0.1-20mM; that is, upto a concentration 5000X that of the Km for $NADP^+$.

D-Glycerate kinase [EC 2.7.1.31].

D-glycerate + ATP ⟶ D 2-phosphoglycerate + ADP

This enzyme was active in cell extracts, being inhibited by Ca^{2+} but activitated by Mg^{2+} ions. Therefore it was assayed in the presence of ethylene glycol-bis(2-amino ethylether)-N,N,N',N'tetraacetic acid (EGTA). The activity was measured in a coupled assay at 40°C containing enolase, which specifically requires D 2-phosphoglycerate not D 3-phosphoglycerate. Addition of the enzyme phosphoglycerate mutase did not increase the rate, suggesting that 2-phosphoglycerate is the product.

Enolase [EC 4.2.1.11].

D 2-phosphoglycerate ⟶ phosphoenolpyruvate + H_2O

The activity recorded required magnesium ions for activity (Km=0.12(±0.02)mM $MgCl_2$). The assay used monitored the formation of the double bond at 230nm.

Pyruvate kinase [EC 2.7.1.40].

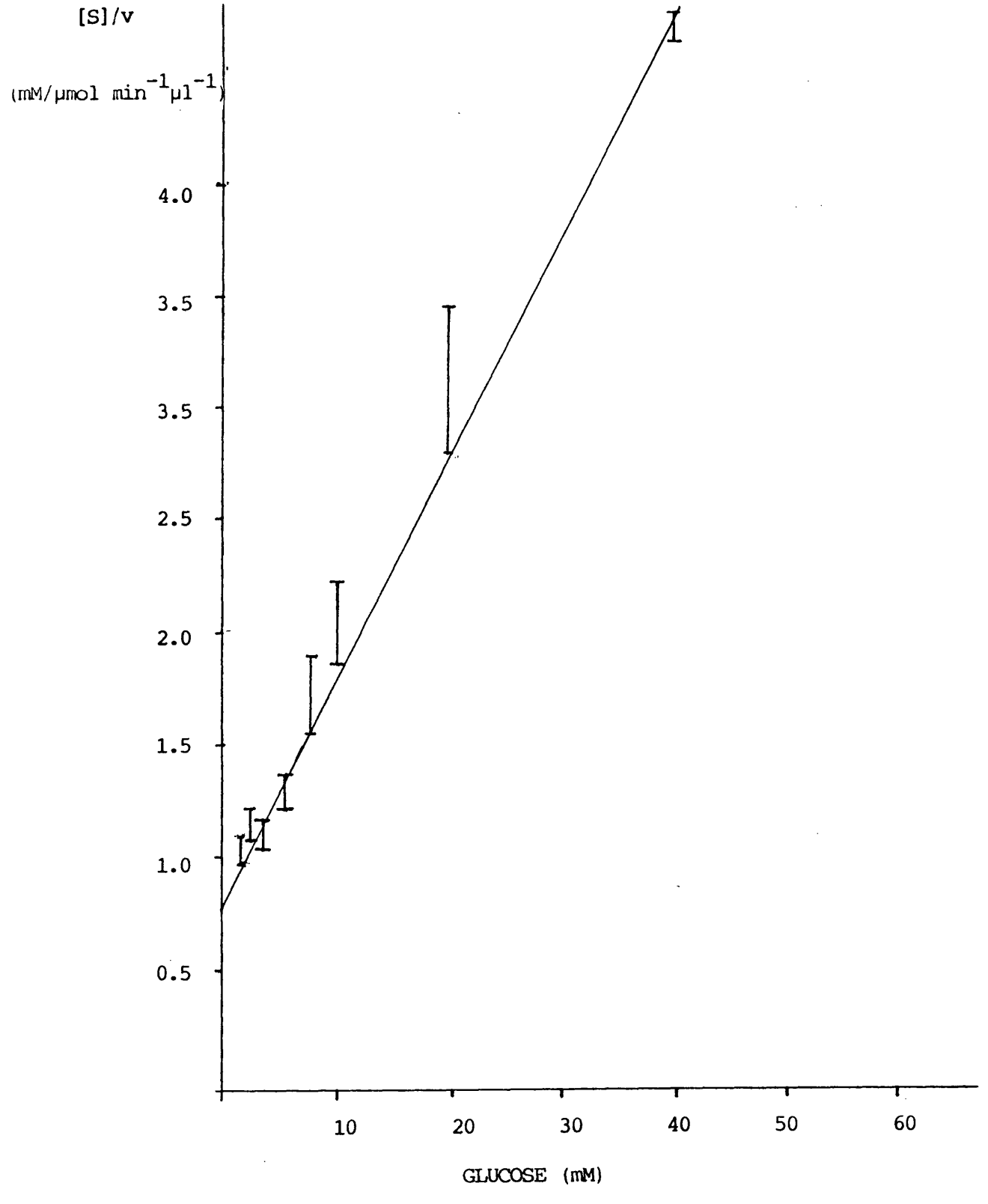
Phosphoenolpyruvate + ADP ⟶ pyruvate + ATP

The activity was assayed by following the formation of pyruvate from phosphoenolpyruvate and ADP. The dependence of enzymic activity on the concentrations of phosphoenolpyruvate was sigmoidal with a Hill coefficent of 1.5.

Undetectable activity

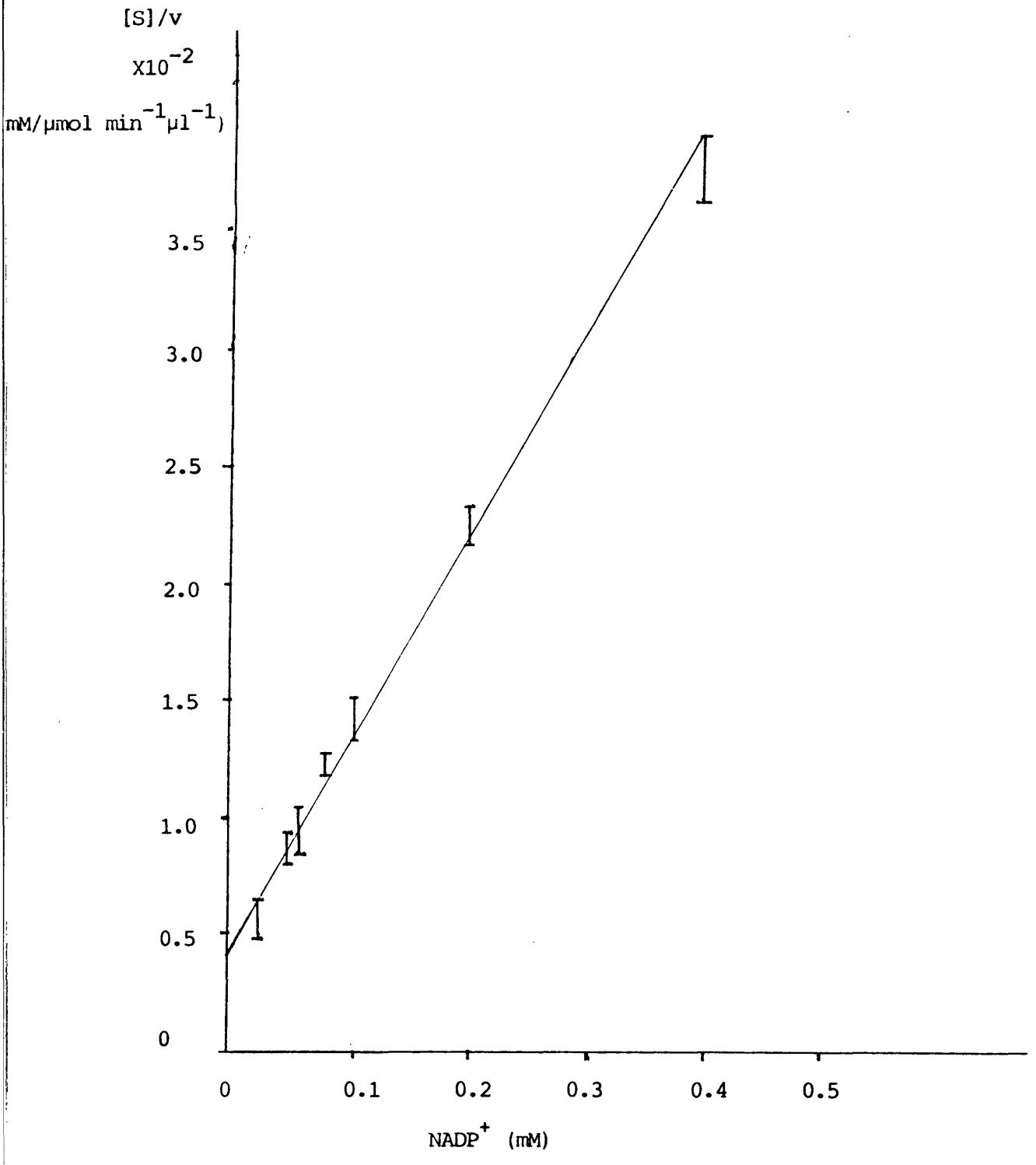
The following enzymes possessed no recordable activity in the cell free extracts of Tp. acidophilum;

Graph 3.2.3. Showing The Half Reciprocal Plot For Glucose Dehydrogenase.



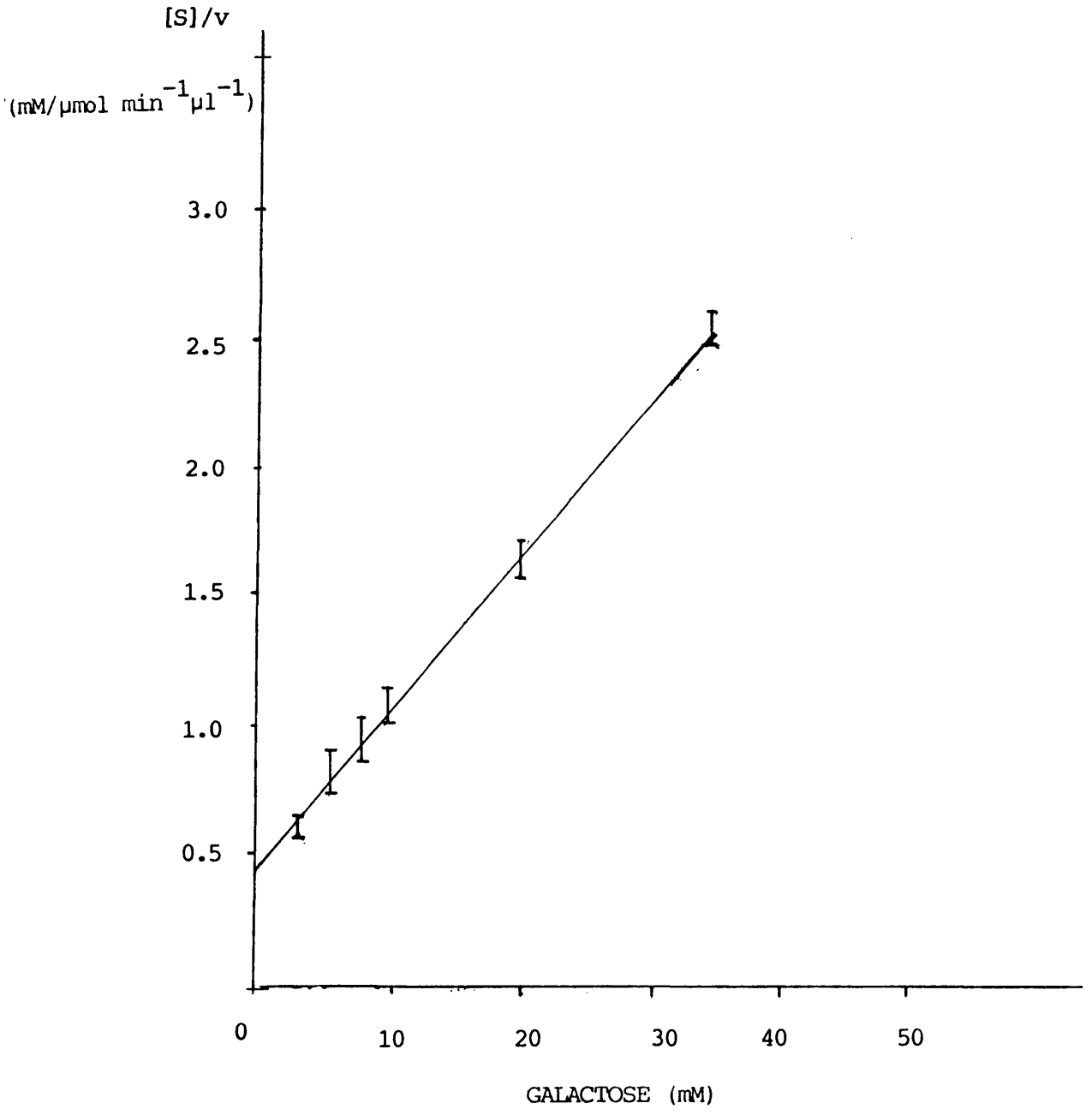
The Km of glucose dehydrogenase for glucose was determined in an assay described in 2.2.5.9 with 0.4mM $NADP^+$. Error bars are the SEM.

Graph 3.2.4. Showing The Half Reciprocal Plot For Glucose Dehydrogenase.



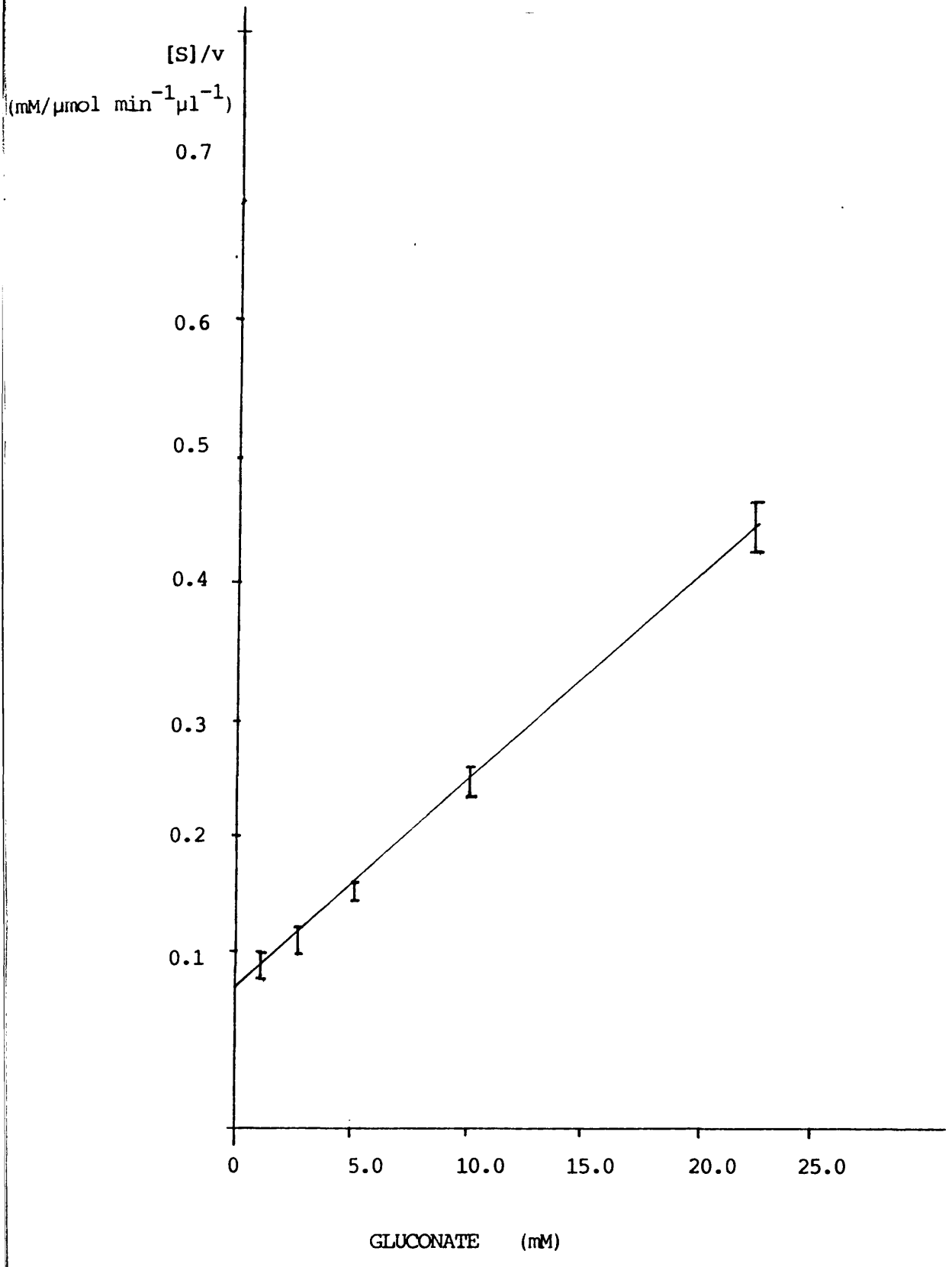
The Km of glucose dehydrogenase for $NADP^+$ was determined in an assay described in 2.2.5.9 with 50mM glucose. Error bars are the SEM.

Graph 3.2.5. Showing The Half Reciprocal Plot For Galactose Dehydrogenase.



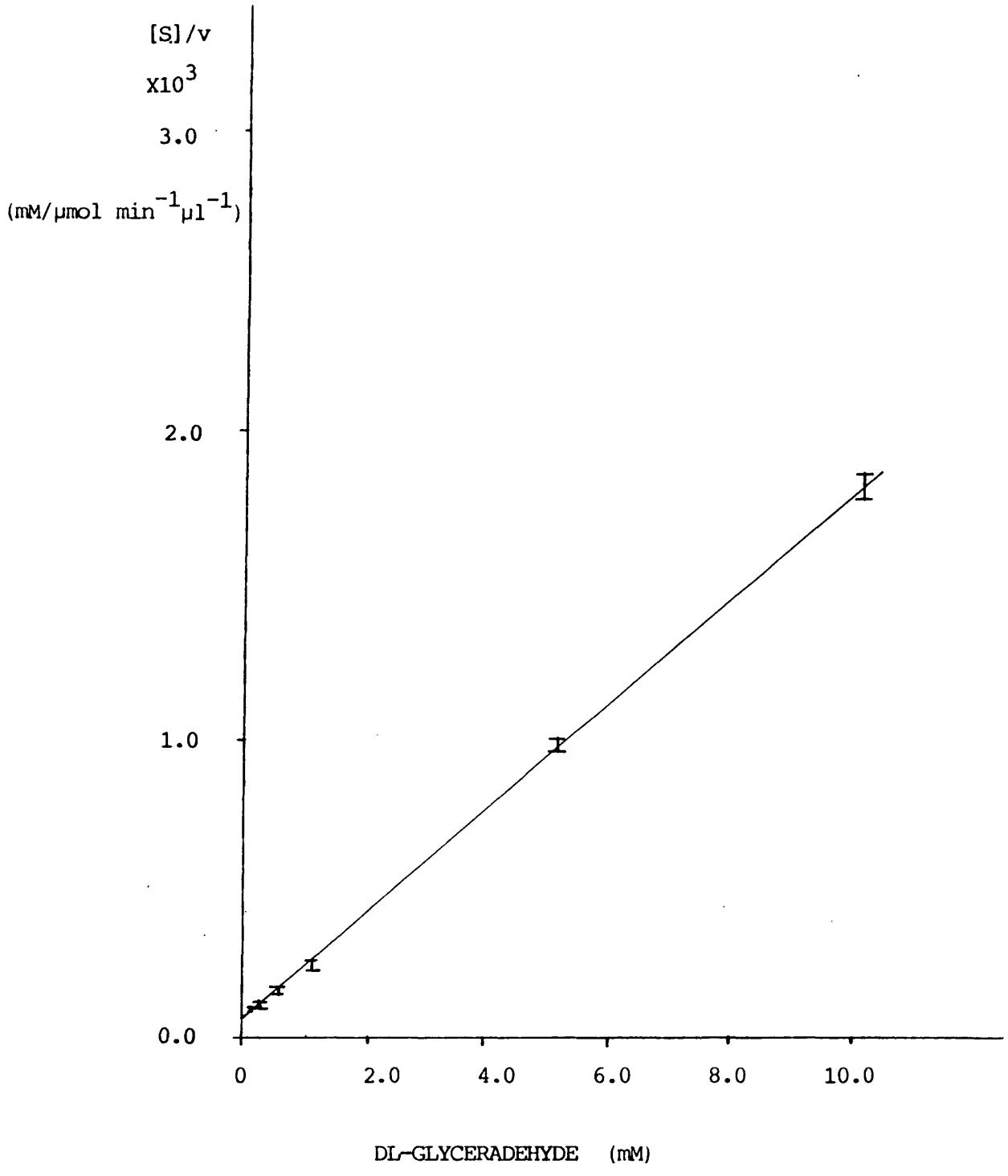
The Km of galactose dehydrogenase for galactose was determined in an assay described in 2.2.5.9 with 0.4mM $NADP^+$. Error bars are the sem.

Graph 3.2.6. Showing The Half Reciprocal Plot For Gluconate Dehydratase.



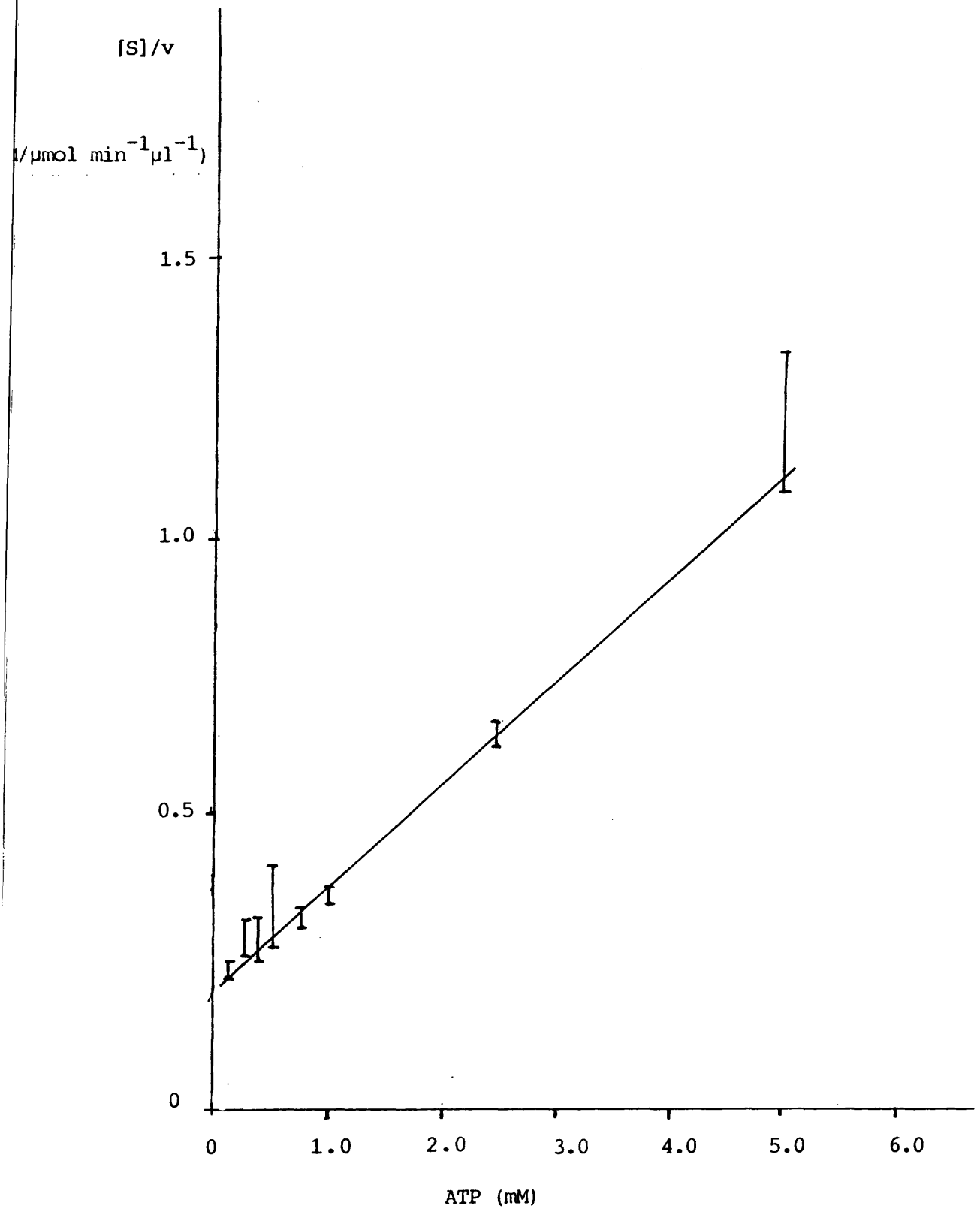
The Km of gluconate dehydratase for gluconate was determined in an assay described in 2.2.5.7. Error bars are the SEM.

Graph 3.2.7. Showing the Half Reciprocal Plot For Glyceraldehyde Dehydrogenase.



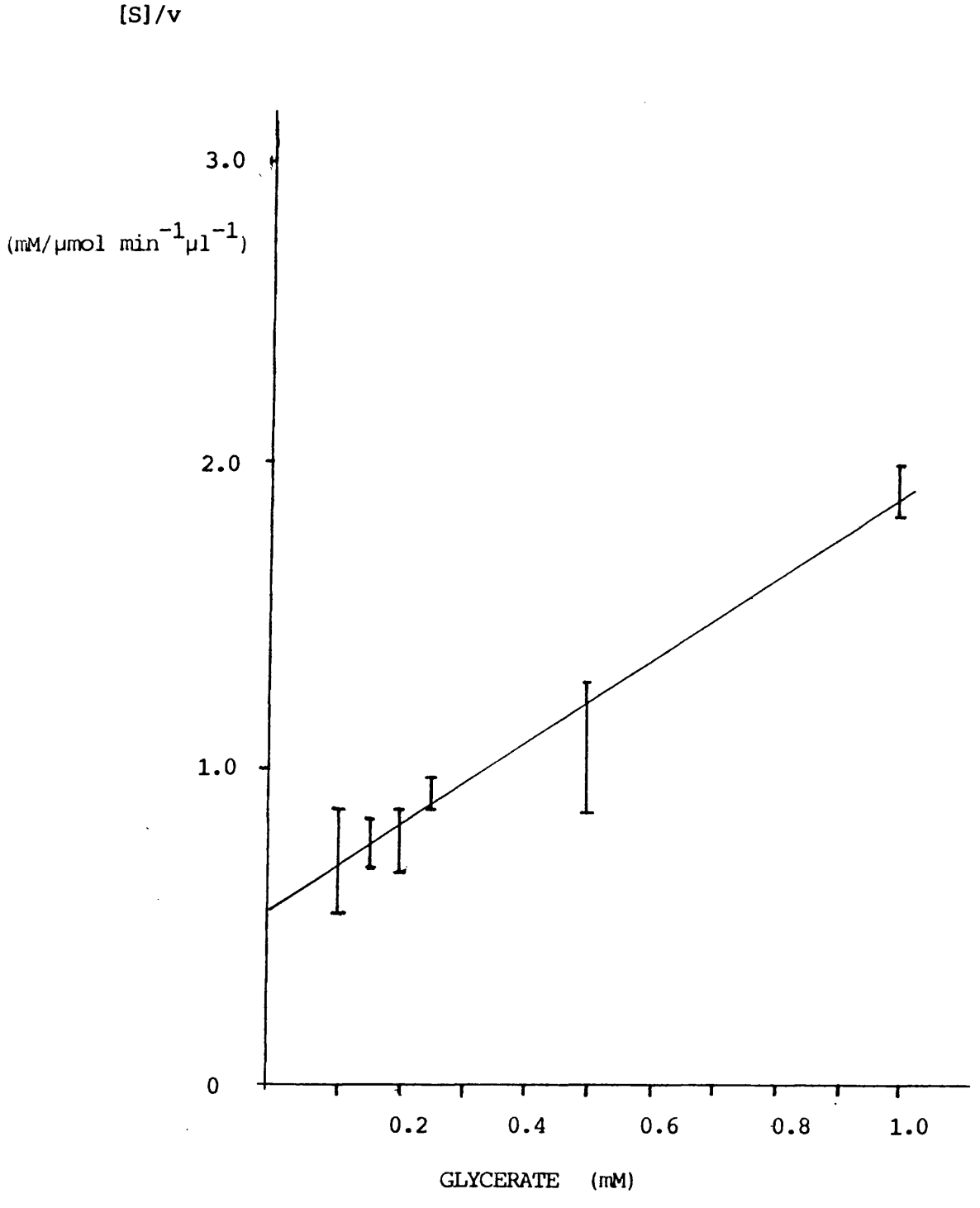
The Km of glyceraldehyde dehydrogenase for DL -glyceraldehyde was determined in an assay described in 2.2.5.7. Error bars are the SEM.

Graph 3.2.8. Showing the Half Reciprocal Plot for Glycerate Kinase.



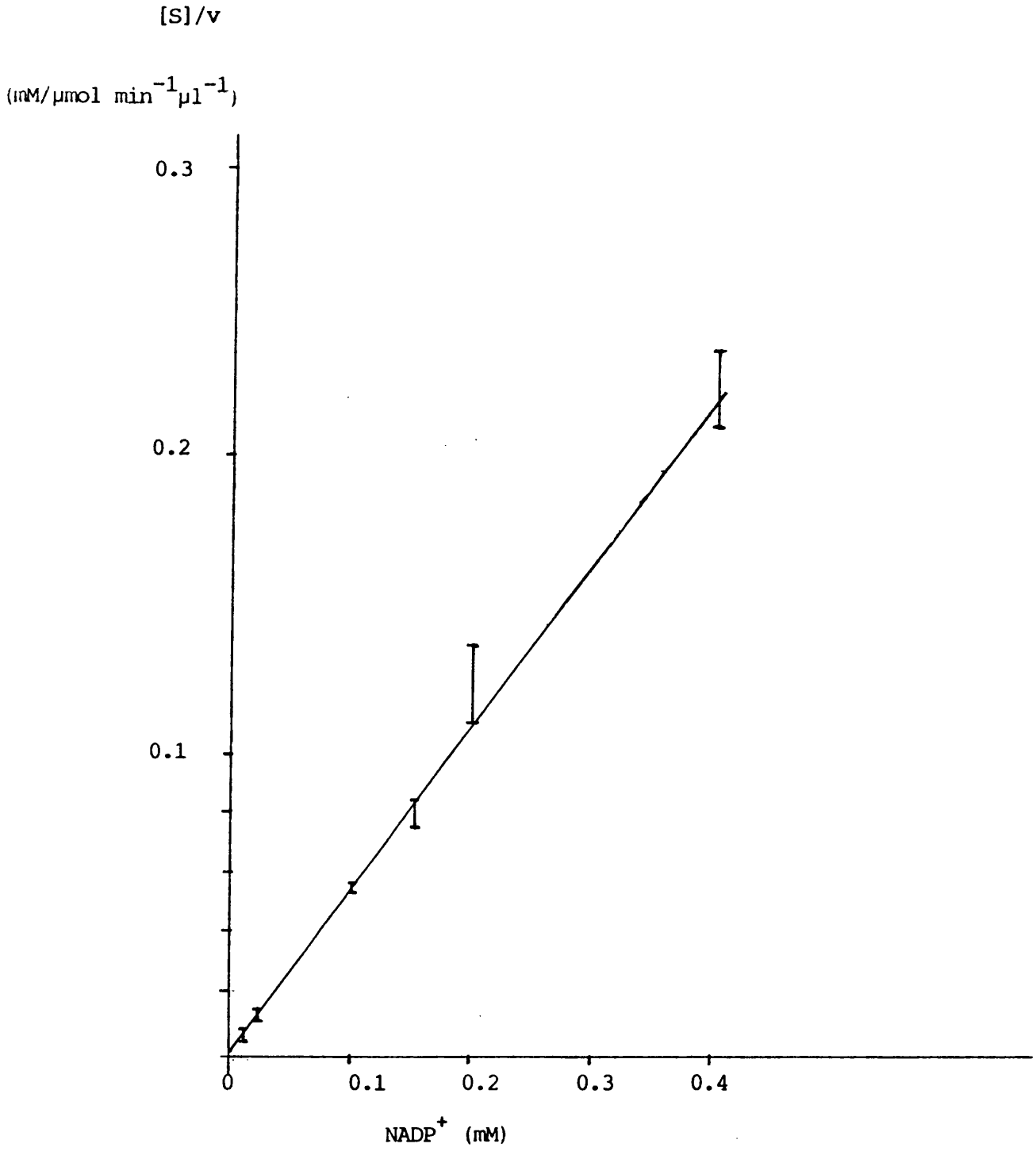
The Km of glycerate kinase for ATP was determined in an assay described in 2.2.5.15. with 5mM DL-glycerate. Error bars are the SEM.

Graph 3.2.9 Showing the Half Reciprocal Plot for Glycerate Kinase.



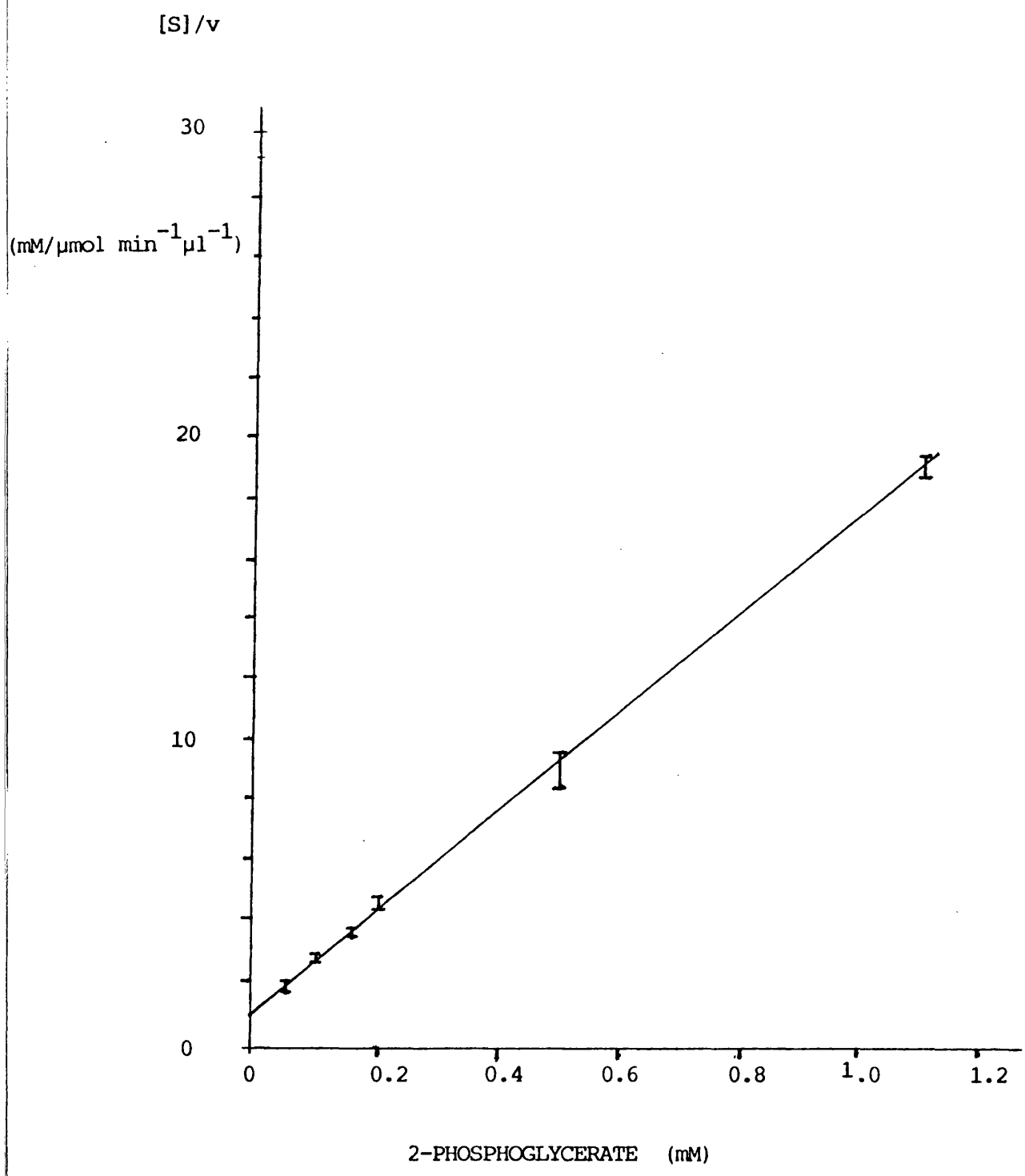
The Km of glycerate kinase for glycerate was determined in an assay described in 2.2.5. with 5mM ATP. Error bars are the SEM.

Graph 3.2.10 Showing the Half Reciprocal Plot for Glyceraldehyde Dehydrogenase.



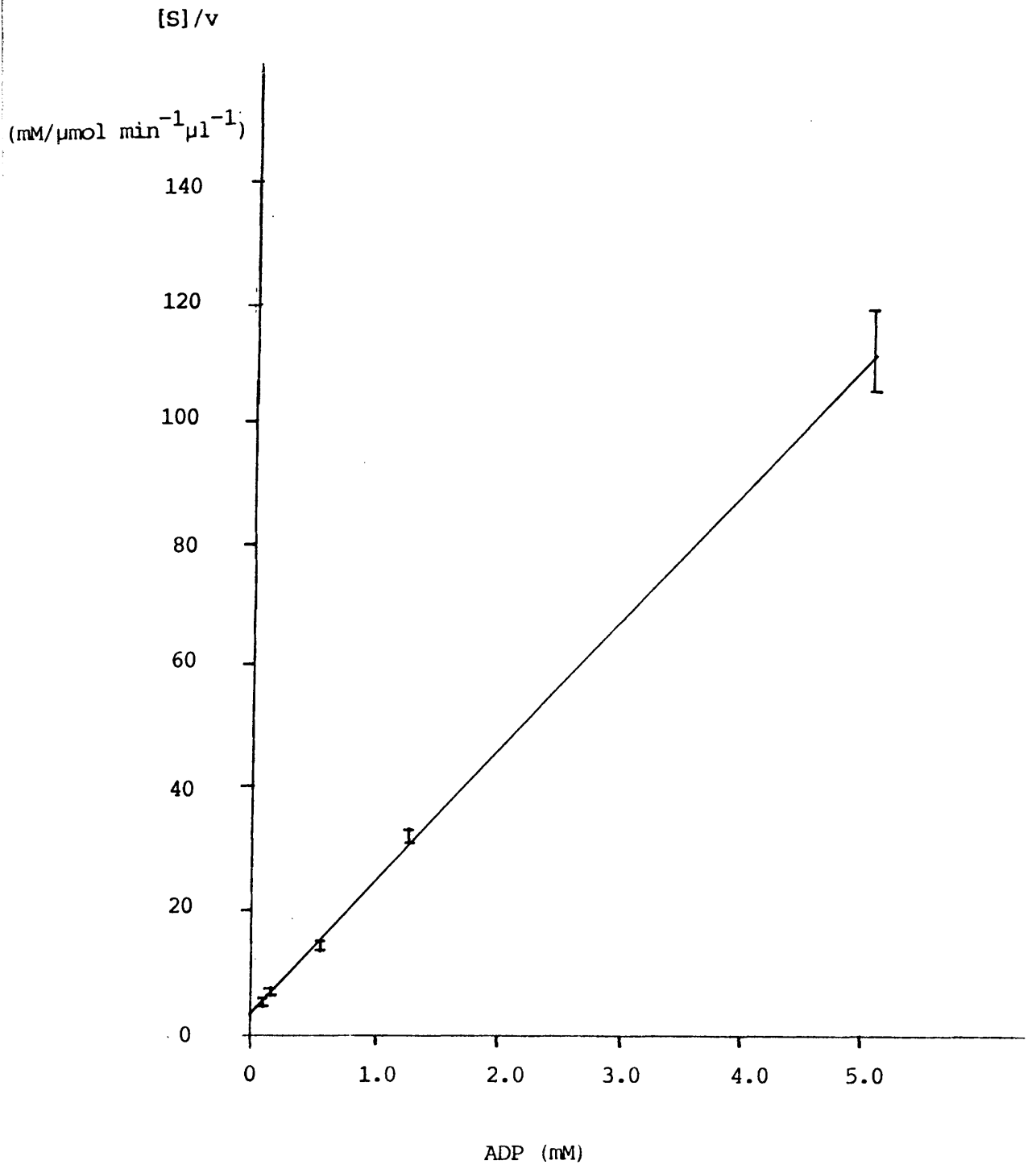
The Km of glyceraldehyde dehydrogenase for $NADP^+$ was determined in an assay described in 2.2.5.11 with 5mM DL-glyceraldehyde. Error bars are the SEM

Graph 3.2.11 Showing the Half Reciprocal Plot for Enolase.



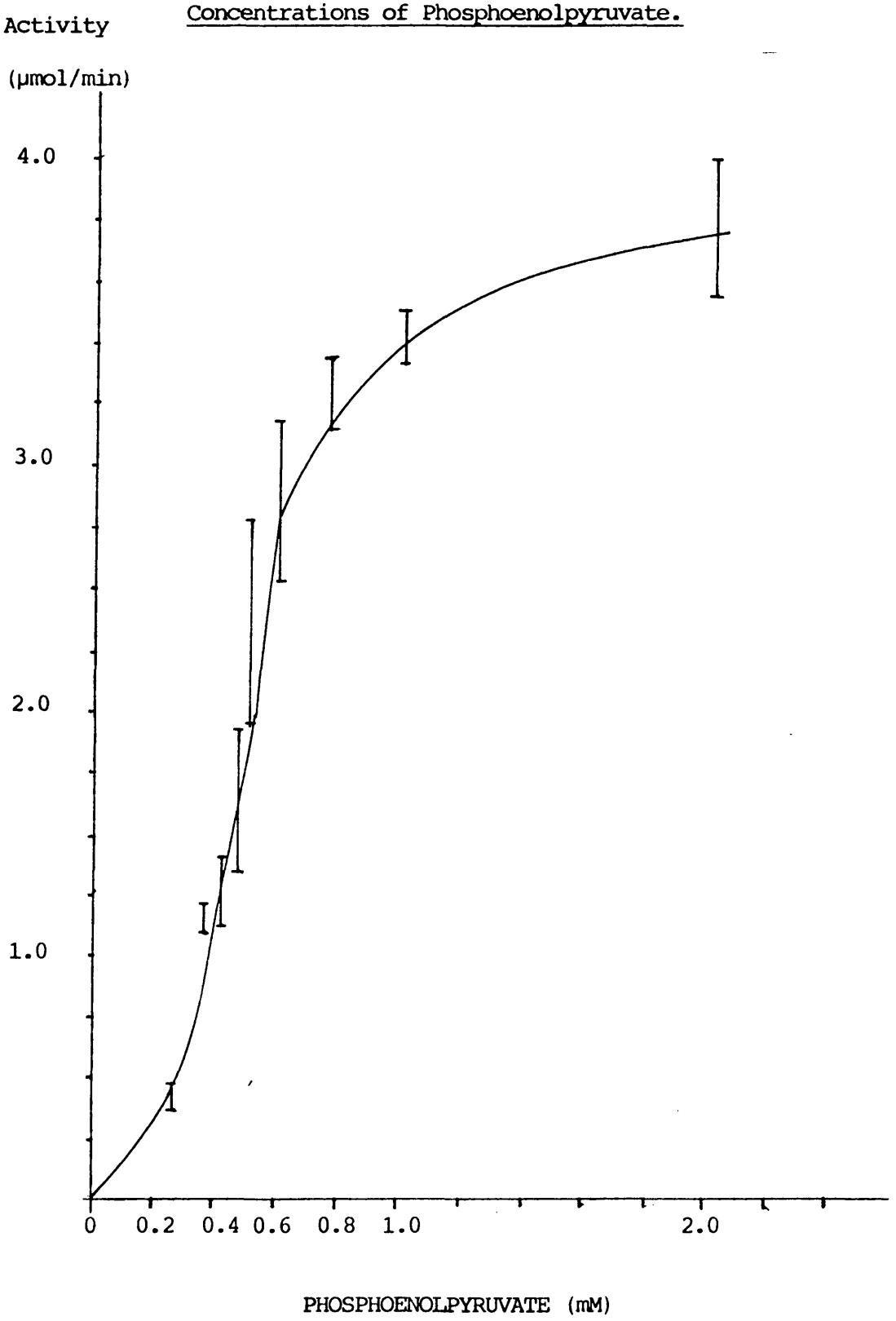
The Km of enolase for 2-Phosphoglycerate was determined in an assay described in 2.2.5.3. The error bars are SEM.

Graph 3.2.12 Showing the Half Reciprocal Plot For Pyruvate Kinase.



The Km of pyruvate kinase for ADP was determined in an assay described in 2.2.5.23. with 10mM phosphoenolpyruvate. Error bars are the SEM.

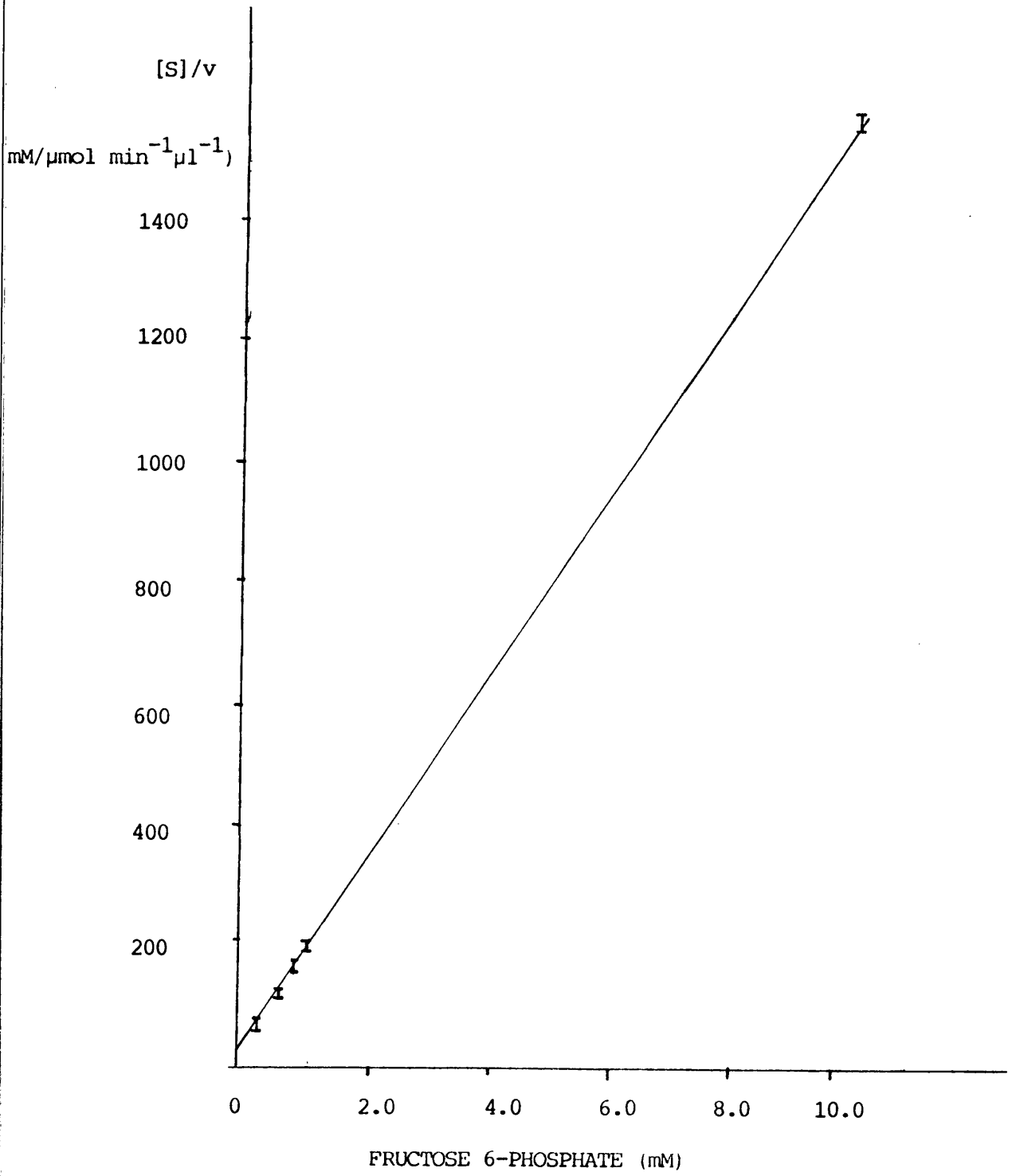
Graph 3.2.13. Showing Pyruvate Kinase Activity With Various Concentrations of Phosphoenolpyruvate.



Pyruvate kinase was assayed as described in 2.2.5.23. with 5mM ADP.

Graph 3.2.14. Showing The Half Reciprocal Plot For Phospho-Glucose Isomerase.

The Km of phosphoglucose isomerase for fructose 6-phosphate was determined in an assay described in 2.2.5.22. Error bars are the SEM.



phosphofructokinase [EC 2.7.1.11], fructose 1,6-bis phosphatase [EC 3.1.3.11], fructose 1,6-bisphosphate aldolase [EC 4.1.2.13], glyceraldehyde 3-phosphate dehydrogenase (assayed with NAD^+ and $NADP^+$) [EC1.1.1.49] and glucokinase [EC 2.7.1.2]. Each of the enzyme assays were verified, at the appropriate temperature, with commercially purified enzymes. FDP-aldolase was assayed for both the formation of fructose 1,6-bisphosphate, and dihydroxyacetone phosphate and glyceraldehyde 3-phosphate with appropriate substrates. Fructose 1-phosphate aldolase was also assayed in the cell extracts without sucess. Glyceraldehyde kinase (triokinase EC 2.7.1.28), which catalyses the phosphorylation of glyceraldehyde was assayed at 40°C for both the formation of ADP and glyceraldehyde 3-phosphate. No recordable activity could be detected in the cell extract in either coupled assay.

Phosphoglycerate mutase [EC 5.4.2.1] catalyses the formation of 2-phosphoglycerate from 3-phospho-glycerate. No activity was detected in the cell extract even with the activitator 1.0mM 2,3-bisphosphoglycerate.

However phosphoglucose isomerase [EC 2.7.5.1] and triose phosphate isomerase [EC 5.3.1.1] activities were found in the cell extract, both being assayed at 40°C with coupling enzymes.

3.2.3 Product analysis

1. Chromatographic detection of pyruvate and glyceraldehyde

0.25mg of a dialysed fresh cell homogenate of Tp. acidophilum was incubated in 100mM Tris/HCl, pH8.0 with the given substrates at 55°C for 90min. The resulting

solutions were reacted with dinitrophenylhydrazine and the dinitrophenylhydrazones produced were identified on silica gel thin layer chromatography plates as described in the methods section.

Summarising the results shown in figure 3.2.15 the following compounds were observed. Pyruvate and glyceraldehyde (distinct from glyceraldehyde 3-phosphate) were produced from gluconate by the enzymatic activities of the cell homogenate. In addition to this, pyruvate was formed from DL-glyceraldehyde, $NADP^+$, ATP, and ADP; however, from DL- glycerate only ATP and ADP were required; D 2-phosphoglycerate and phosphoenolpyruvate required only ADP to form pyruvate. Pyruvate, however was not formed from the glycolytic intermediates D 3-phosphoglycerate and DL-glyceraldehyde 3-phosphate under conditions that would be expected to yield the keto acid if the appropriate enzyme activities were active.

2. Chromatographic Identification of 2-keto 3-deoxygluconate

Kersters & De Ley (b. 1968) showed that 2-keto 3-deoxygluconate, when reacted with o-phenyldiamine, gives a light yellow fluorescent spot with an Rf of 0.38 when developed on silica gel thin layer chromatography plate run in n-propanol/ammonia .88/0.2%(w/v) EDTA (6;3;1). This citerian was used by De Rosa *et al.* (1984) for the identification of the compound and is also used here.

Figure 3.2.16 shows the R_f values of fluorescent spots on silica gel thin layer chromatography plates after 0.5mg of the cell homogenate was incubated with and without

Figure 3.2.15a. Chromatographic detection of pyruvate.

0.25mg of a cell extract of Tp. acidophilum were incubated in 100mM Tris/HCl, pH8.0, with the substrates described for 90min at 55°C. The solutions were reacted with dinitrophenylhyrazine, extracted into ethylacetate and applied to silica gel TLC plates and developed in butanol/ ammonia 0.88 / ethanol (6:2:1). The R_f values of the yellow dinitrophenylhydrazones separated are given with the various incubation mixtures.

Incubation mixtures	R_f values of DNP-hydrazones		
Pyruvate (standard)	0.33	0.49	
0.25mg cell extract (control)			0.92
10mM glycerate, 5mM ATP, 5mM ADP, 10mM KCl, 10mM EGTA, 10mM $MgCl_2$	0.32	0.49	0.93
10mM D 2-phosphoglycerate, 5mM ADP, 10mM $MgCl_2$, 10mM KCl	0.31	0.49	0.92
10mM phosphoenolpyruvate, 5mM ADP, 10mM $MgCl_2$,10mM KCl	0.31	0.49	0.92
10mM D 3-phosphoglycerate, 5mM ADP, 10mM $MgCl_2$, 10mM KCl.			0.93
3.3mM DL-glyceraldehyde 3-phosphate, 5mM ATP, 5mM ADP, 10mM $MgCl_2$, 10mM KCl, 0.4mM $NADP^+$, 0.4mM NAD^+, 10mM $KHPO_4$.	0.00		0.91

Figure 3.2.15.b Chromatographic detection of pyruvate and glyceraldehyde.

0.25mg of a cell extract of Tp. acidophilum were incubated in 100mM Tris/HCl, pH8.0, with the substrates described for 90min at 55°C. The solutions were reacted with dinitrophenylhydrazine, extracted into ethylacetate and applied to silica gel TLC plates and developed with ethylacetate /acetone /ethanol (1:1:1). The R_f values of the yellow dinitrophenylhydrazones separated are given with the various incubation mixtures.

INCUBATION MIXTURES	R_f DNP-HYDRAZONES			
Glyceraldehyde (standard)				0.91
Pyruvate (standard)	0.41	0.79		
0.25mg of cell extract only				1.0
0.25mg of cell extract, 50mM D-glucose 2mM $NADP^+$, 20mM $MgCl_2$.	0.45	0.83		1.0
0.25mg of cell extract, 50mM gluconate 20mM $MgCl_2$	0.41	0.83	0.91	1.0
0.25mg of cell extract, 10mM $MgCl_2$ 10mM DL-glyceraldehyde, 10mM KCl, 5mM ATP, 5mM ADP, and 2mM $NADP^+$.	0.43	0.81	0.90	

Figure 3.2.16. Chromatographic Identification of 2-Keto 3-deoxy-gluconate.

0.5mg of a cell extract of Tp. acidophilum was incubated in 100mM Tris/HCl, pH8.0, containing 50mM D-gluconate and 20mM $MgCl_2$ at 55°C for 60min. After being dried down, the residue was applied to a silica gel TLC plate and developed in n-propanol/ammonia/0.2%(w/v) EDTA (6:3:1 by volume). The R_f values and colours of the fluorescent spots are given when viewed under ultra-violet light after spraying the TLC plates with o-phenylenediamine in 10%(w/v) trichloroacetic acid and heated at 105°C.

INCUBATION MIXTURE	R_f VALUES AND COLOUR OF FLUORESCENT SPOTS
Glyceraldehyde (standard)	0.64 Green
Pyruvate (standard)	0.36 Green 0.68 Green
0.5 mg cell extract	0.28 0.58 0.85
0.5 mg cell extract, 50mM D-gluconate and 20mM $MgCl_2$.	0.26 0.35 Yellow 0.44 Green 0.57 0.70 Green 0.86

50mM gluconate and 20mM $MgCl_2$ in 100mM Tris/HCl, pH8.0, at 55°C for 60min. As described in figure 3.2.16 a light yellow spot was identified with an R_f value of 0.35, obtained under conditions described by Kersters & De Ley (1968). This suggests that 2-keto 3-deoxygluconate is formed from D-gluconate by the cell extract.

3.2 Enzymatic analysis of products.

The consumption of glucose and the production of NADPH pyruvate, D-glyceraldehyde and D-glyceraldehyde 3-phosphate were monitored enzymatically after incubations of Tp. acidophilum cell extracts with D-glucose, potassium gluconate, DL-glyceraldehyde and various cofactors. The stoichiometry of the products and the incubation conditions are given in table 3.2.17.

Summarising the results, for every mol of glucose consumed 1mol of NADPH is produced during the production of gluconate. Pyruvate and D-glyceraldehyde are produced from gluconate in equal amount, whereas D-glyceraldehyde 3-phosphate was not produced in any significant quantity with or without addition of ATP. Glyceraldehyde was further metabolised to equal molar ratio of NADPH and pyruvate. Glucose was catabolised without ATP or ADP to 2NADPH's and, within experimental error, 1 pyruvate. However, with ATP and ADP 2 pyruvates were formed, giving equal amounts of NADPH and pyruvate produced overall.

Figure 3.2.17. Stoichiometry of Products

Cell extracts of Tp. acidophilum were incubated as outlined below and the resulting products produced and substrates consumed were determined by enzymatic analysis given in section 2.2.7. Errors are the SEM of 4 experiments.

Incubation conditions (at 55°C in 100mM Tris/HCl, pH8.0)	Stoichiometry of Products
0.25mg of cell extract, 0.1mM glucose and 0.4mM $NADP^+$	Glucose (consumed/NADPH (produced) 1.0 : 0.90(±0.36)
0.1mg of cell extract, 50mM K^+gluconate 10mM $MgCl_2$	Pyruvate (prod.) /Glyceraldehyde 1.0 :1.0(±0.4) Pyruvate (prod.) /Glycer'de 3-phos. 1.0 : 0.11(±0.18)
0.1mg of cell extract, 50mM K^+gluconate 1.0mM ATP and 10mM $MgCl_2$.	Pyruvate (prod.) /Glycer'de 3-phos. 1.0 : 0.10(±0.15)
0.1mg of cell extract, 5mM glyceraldehyde, 0.16mM $NADP^+$, 5mM ATP, 5mM ADP, 10mM $MgCl_2$ and 10mM KCl.	NADPH (produced) /Pyruvate 1.0 : 1.12(±0.26)
1.2mg of cell extract, 0.05mM glucose 0.4mM $NADP^+$, and 10mM $MgCl_2$.	Glucose (consumed) /NADPH (prod.) 1.0 : 1.70(±0.02) Glucose (consumed) /Pyruvate (prod.) 1.0 : 0.58(±0.1)
1.2mg of cell extract, 0.05mM glucose 0.4mM $NADP^+$, 5mM ATP, 5mM ADP, 10mM $MgCl_2$ and 10mM KCl.	Glucose (consumed) /Pyruvate (prod.) 1.0 : 1.3(±0.4) NADPH (produced) /Pyruvate (prod.) 1.0 : 0.78(±0.24)

3.3. Discussion

3.3.1. Hexose requirement

Tp. acidophilum is capable of growing on glucose, galactose, or gluconate but not glycerol. Growth on gluconate is particularly interesting since it is supported by the enzymatic activities and product identification results. Similarly growth on galactose can also be rationalised in view of the discovery of galactose dehydrogenase.

3.3.2. Enzymatic activities and product analysis.

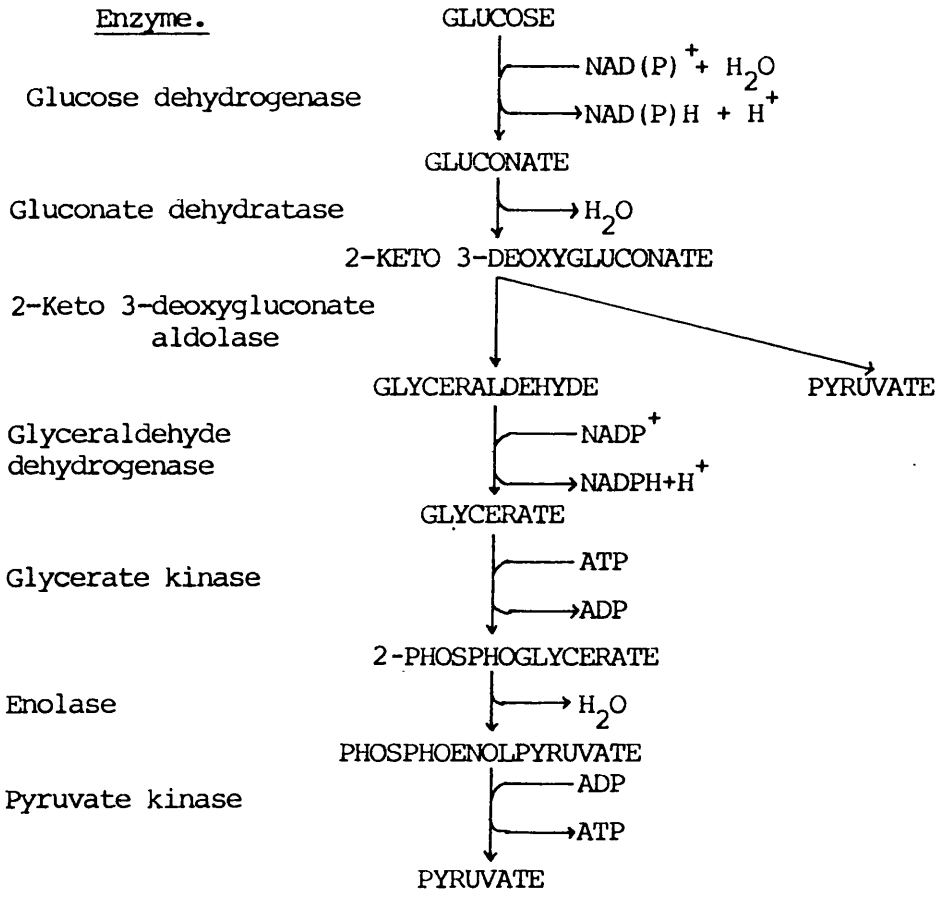
The data obtained are consistent with a novel catabolic pathway which involves no phosphorylated hexoses(figure 3.3.1). The scheme proposed is based on the enzymatic activities found, the intermediates identified and the stoichiometeriès of the end products obtained.

The activities of glucose dehydrogenase, gluconate dehydratase and KDG aldolase catalyse the conversion of glucose to equimolar amounts of glyceraldehyde and pyruvate. Since glyceraldehyde kinase or triokinase were not detected but glyceraldehyde dehydrogenase was found, the glyceraldehyde formed was oxidised to glycerate rather than being phosphorylated. Glycerate kinase then phosphorylates the glycerate to 2-phosphoglycerate which is further metabolised to a second molecule of pyruvate by enolase and pyruvate kinase.The enzyme KDG-aldolase is the key and unique activity of this novel pathway.

Glucose, gluconate, glyceraldehyde, glycerate, 2-phosphoglycerate and phosphoenolpyruvate all produced

Figure 3.3.1 Proposed Pathway of Glucose Catabolism in Tp. acidophilum.

The enzymatic and product analysis is consistent with the following non-phosphorylated Entner-Doudoroff pathway.



pyruvate when incubated with cell free extract and the cofactors ATP, ADP and $NADP^+$, thereby identifing these C6 and C3 compounds as intermediates of the pathway. 2-Keto 3-deoxygluconate was synthesized from gluconate by the cell extracts, identifying it as another intermediate. All these findings are satisfied by the proposed pathway.

On the basis of key activities and product analysis from known intermediates, the Embden Meyerhof pathway does not appear to operate in this organism. Pyruvate was not formed from glyceraldehyde 3-phosphate or D 3-phosphoglycerate, consistent with the observed absence of glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate mutase activities in the extracts. The absence of phosphofructokinase, glucokinase and FDP-aldolase further supports the absence of the Embden Meyerhof pathway. Since fructose 1,6-bisphosphatase and an fructose 1,6-phosphate-forming aldolase were not recorded, the possibility of the conventional gluconeogenic pathway also seems unlikely.

Two enzymes of the Embden Meyerhof pathway were found namely phosphoglucose isomerase and triose phosphate isomerase. The function of phosphoglucose isomerase in Tp. acidophilum is not clear, especially since glucose 6-phosphate dehydrogenase activity was not found. The enzyme may be involved in the metabolism of fructose to glucose via a phosphorylated hexose as found in Sulfolobus solfataricus (De Rosa et al., 1984). Perhaps more puzzling was the detection of triose phosphate

isomerase activity, an enzyme which appears to be met metabolically 'isolated' as the pathway stands. Further investigation is required to identify the role of this enzyme in Tp. acidophilum.

The classical Entner-Doudoroff pathway also appeared absent, since glucose 6-phosphate dehydrogenase 1.1.1.49) activity was not detected and incubation with 6-phosphogluconate, or gluconate and ATP, yielded no glyceraldehyde 3-phosphate. Searcy & Whatley (1984) support this as they were unable to show 6-phospho-gluconate dehydratase or 2-keto 3-deoxyphosphogluconate aldolase activity in extracts of Tp. acidophilum . However, on the basis of respiratory activities with various substrates, they concluded that the Embden-Meyerhof pathway and pentose phosphate pathway are active in Tp. acidophilum. They reported respiratory activities with DL-glyceraldehyde and gluconate, and also showed that gluconate (gluconolactone) was one of the first major products when growing cells were spiked with ^{14}C-glucose. No phosphorylated hexoses were observed These findings support the existence of the novel pathway proposed in this thesis.

The proposed pathway has been constructed on evidence obtained with cell extracts of Tp, acidophilum prepared by sonication. To verify the existence of this pathway in growing cells and to resolve the apparently contradictory evidence provided by Searcy & Whatley (1984) on the presence of the Embden-Meyerhof pathway, radio-respirometeric analysis was used. This work is described

in chapter 5.

The overall stoichiometry of the pathway is;

Glucose + 2 $NADP^+$ ⟶ 2 Pyruvates +2 $NADPH+2H^+$

There is no net ATP synthesis and there is no regeneration of reducing equivalents. In the Embden-Meyerhof pathway 2ATPs are formed for every glucose oxidised as far as pyruvate, compared with one in the Entner-Doudoroff pathway. This novel pathway in Tp. acidophilum lacks the almost universal sequence of reactions which convert D-glyceraldehyde 3-phosphate to pyruvate and yield two ATP's. Additional enzymes were assayed to search for a metabolic route effecting a net ATP synthesis and a possible fermentative pathway. The results of this study are also given in chapter 5.

Another interesting problem that deserved investigation was the route of glycerol synthesis, and its relation to this novel pathway. This study is dealt with in chapter 4.

The pathway found in Tp. acidophilum is compared with those of other archaebacteria and of eubacterial and eukaryotic species in chapter 7.

CHAPTER 4.

GLYCEROL SYNTHESIS

4.1 Introduction.

Archaebacterial lipids differ from those of the other two primary kingdoms in a number of respects. They consist of isoprenoid and hydroisoprenoid hydrocarbons, they possess an ether bond between the glycerol backbone and the isoprenoid alcohol, and the optical rotation of the central carbon of the glycerol is opposite to that found in eubacteria and eukaryotes (Langworthy, 1985).

The synthesis of archaebacterial lipids has been partially studied. The formation of the isoprenoid alcohols has been worked out in principle (Langworthy,1985), although the synthesis of glycerol or glycerol-phosphate and the ether bond formation has yet to be fully elucidated.

Eukaryotes and eubacteria possess membrane lipids with ester bonds between the glycerol and fatty acid, but some eukaryotes also possess non-membrane lipids with an ether bond and ester bond such as plasminogens.

The glycerol moiety in the ester-lipids of eukaryotes and eubacteria is derived from (sn)-glycerol 3-phosphate which is synthesised by the stereochemically specific (sn)-glycerol 3-phosphate dehydrogenase [EC 1.1.1.8.]. However, the 'glycerol backbone' in the ether lipids of eukaryotes is derived from dihydroxyacetone phosphate rather than (sn)-glycerol 3-phosphate. The ether bond is thought to be synthesised as shown in figure 4.1.1:

a) dihydroxyacetone phosphate forms an ester with acyl-CoA giving 1-acyldihydroxyacetone phosphate;b) an exchange occurs between the acyl group and a long chain alcohol - resulting in an ether bond and c) the 1-alkyl-dihydroxy-actone phosphate is reduced to 1-alkylglycerol 3-phosphate, which is further metabolised to form plasminogens or ether acylglycerols.

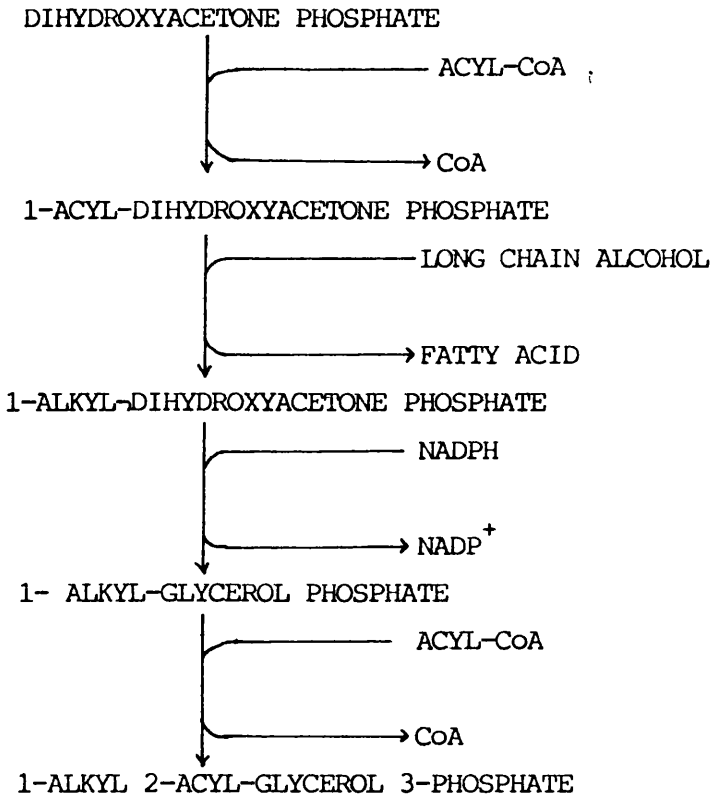
Since the opposite isomer of glycerol is found in the archaebacterial membrane ether lipids, being substituted in positions 2 and 3, it was of interest to discover the route of its synthesis is Tp. acidophilum and how it relates to the discovered pathway of glucose catabolism.

De Rosa et al.(1986) have shown that the incorporation of glycerol into the backbone of the lipids of Sulfolobus acidocaldarius occurs without change in the hydrocarbon skeleton. They suggested that glycerol/glycerol phosphate (unknown stereoisomer) is the biosynthetic precursor and that the process does not proceed via a keto or aldol intermediate such as dihydroxyacetone phosphate as in eukaryotic ether lipids. They proposed that the glycerol/ glycerol phosphate is alkylated by geranyl geranyl - pyrophosphate to give the stereochemical form of the lipid. No enzymatic activities were identified.

Using extracts of S. acidocaldarius and Tp. acidophilum assays were performed to demonstrate enzymatic activities that would generate suitable stereochemical forms of

Figure 4.1.1. Biosynthesis of ether lipids in eukaryotes and eubacteria.

The biosynthesis of lipids possessing an ether bond, such as found in plasminogens, is thought to consist of the following set of reactions starting with the precursor dihydroxyacetone phosphate.



glycerol from glyceraldehyde, an intermediate of glucose metabolism. Since Tp. acidophilum possesses a novel pathway of glucose metabolism and the glycerol 'backbone' has a (sn) 2,3 stereochemical configeration, it would be expected that the biosynthesis of the triose will be different from that found in the eubacteria and eukaryotes.

4.2. Results.

4.2.1 Enzymatic activities.

The scheme outlined in figure 4.2.1. describes the possible synthetic routes to produce glycerol or DL-glycerol phosphate from DL-glyceraldehyde, an intermediate in glucose catabolism in Tp. acidophilum. All enzymes shown were assayed in cell extracts of Tp. acidophilum and S. acidocadarius.

a) Glyceraldehyde reductase (glycerol:$NADP^{+}$oxidoreductase [EC 1.1.1.72.]).

DL-glyceraldehyde + NADPH + H^{+} $\longleftrightarrow$ glycerol + $NADP^{+}$

Glyceraldehyde reductase was detected in both Tp. acidophilum and S. acidocaldarius cell extracts. The Km values calculated from the direct linear plot analysis of Eisenthal & Cornish-Bowden (1974), and the specific activities at 55°C for both enzymes are given in table 4.2.2.. The data for both enzymes are presented graphically as half reciprocal plots in figures 4.2.3. -4.2.6.

In both organisms the DL-glyceraldehyde reductase was also assayed with the formation of glyceraldehyde from glycerol. The specific activities in this'reverse'direction were for Tp. acidophilum 4.6 $nmoles\,min^{-1}mg^{-1}$ and for S. acidocaldarius 2.2 $nmoles\,min^{-1}mg^{-1}$. Mg^{+2} ions and 2-mercaptoethanol were required by both enzymes for full activity.

b) (sn)-Glycerol 3-phosphate dehydrogenase [EC 1.1.1.8.] NAD^{+}dep and EC 1.1.1.94. for $NADP^{+}$dep].

dihydroxyacetonephosphate+NAD(P)H+H^{+} $\longrightarrow$ (sn)-glycerol 3-pho'te +NAD(P)$^{+}$

Assays were performed with cell extracts of both

Figure 4.2.1. Possible routes of synthesis of glycerol or glycerol phosphate.

The scheme shows the possible routes of glycerol or glycerol-phosphate synthesis for lipid biosynthesis.

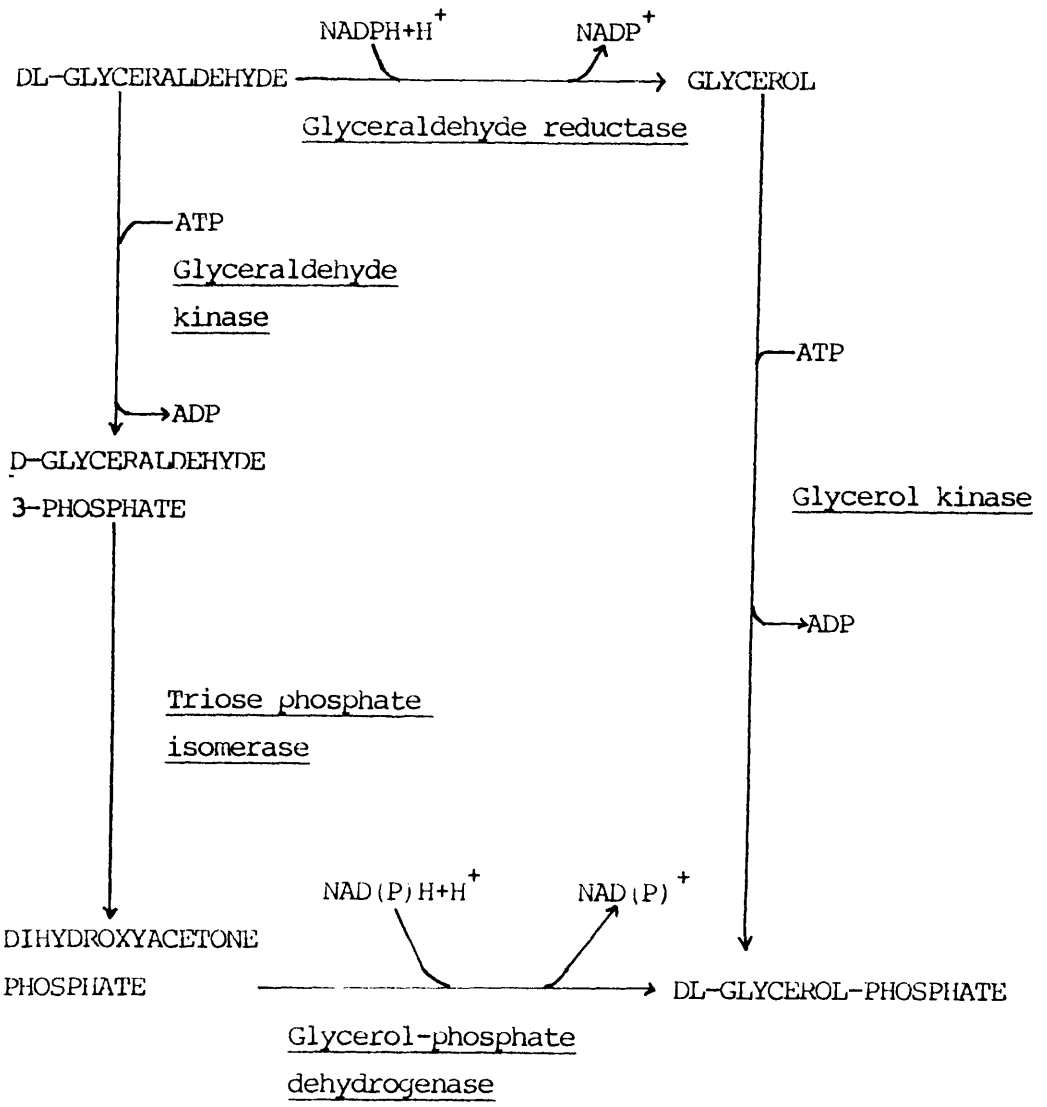
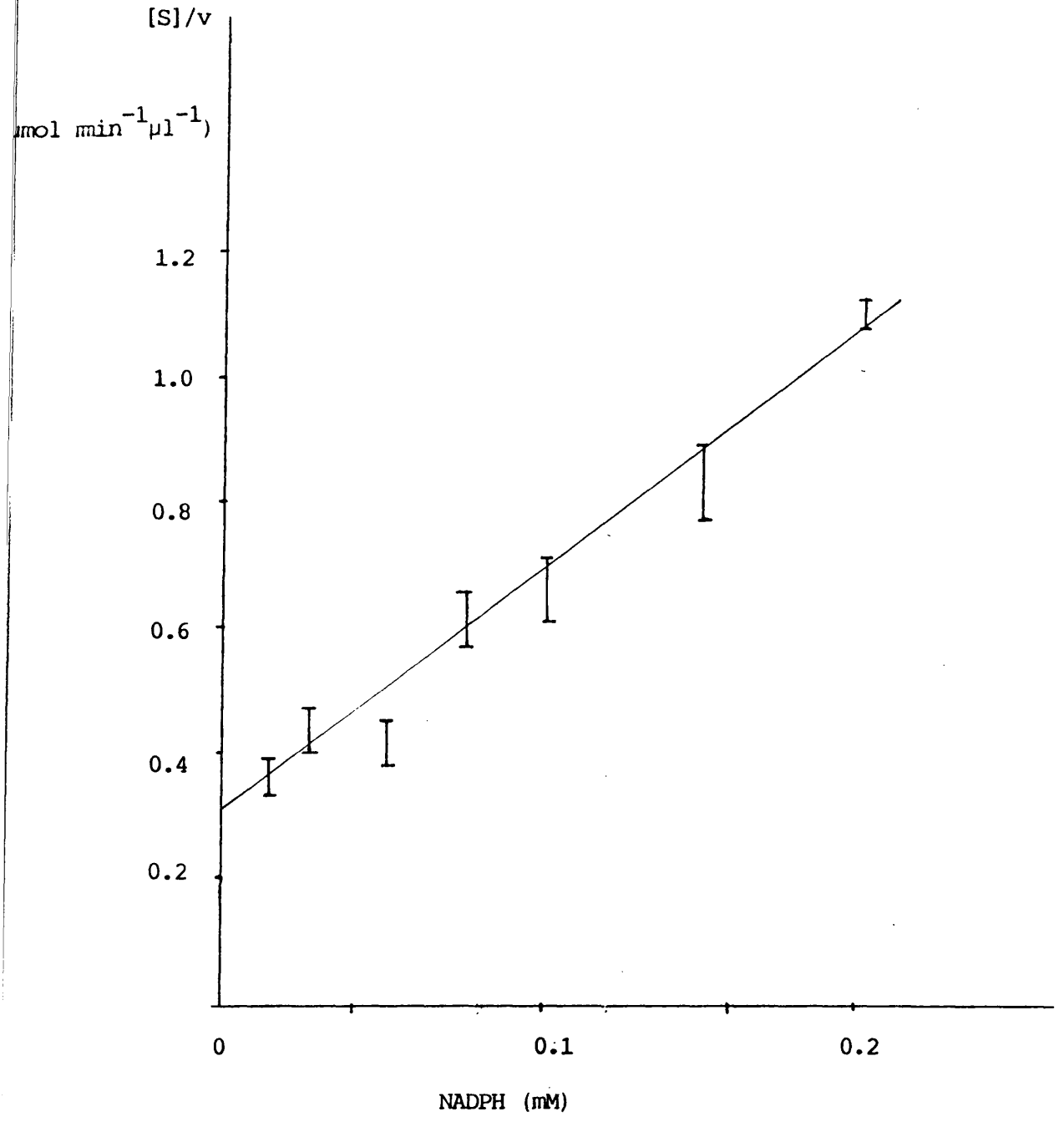


Table 4.2.2. Enzyme kinetic parameters for glyceraldehyde reductase[EC 1.1.1.72.]

SOURCE	ASSAY TEMP. (°C)	SPECIFIC ACTIVITY (nmol $min^{-1}mg^{-1}$)	Km VALUES (mM)	
Tp.acidophilum	55	9.51 (±0.58)	NADPH	0.047(±0.0095)
			DL-glyceraldehyde	9.00(±1.35)
S.acidocaldarius	55	16.65(±1.61)	NADPH	0.077(±0.021)
			DL-glyceraldehyde	22.7(±4.5)

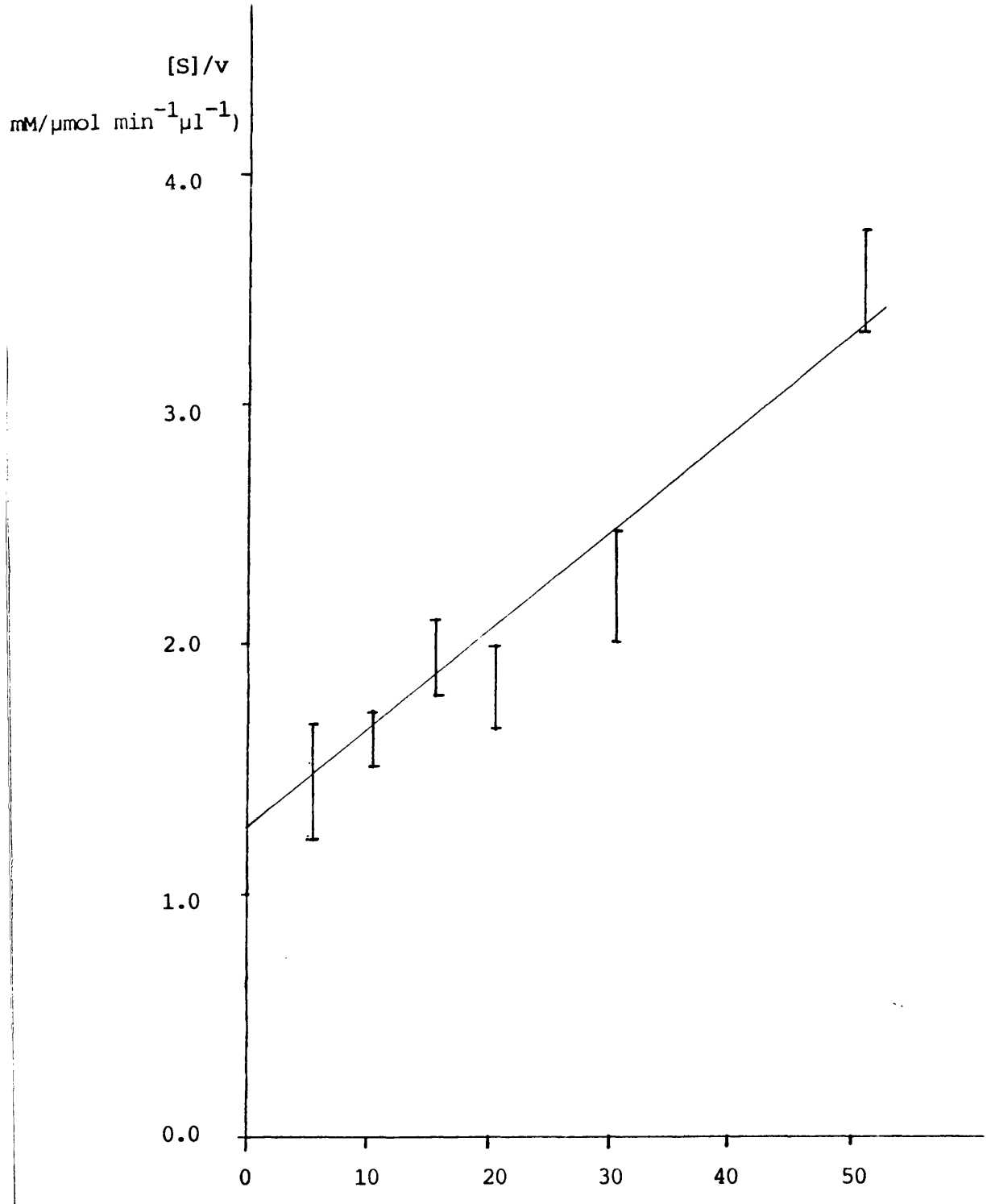
Errors are SEMs.

Graph 4.2.3. Showing The Half Reciprocal Plot For Glyceraldehyde Reductase From Sulfolobus acidocaldarius.



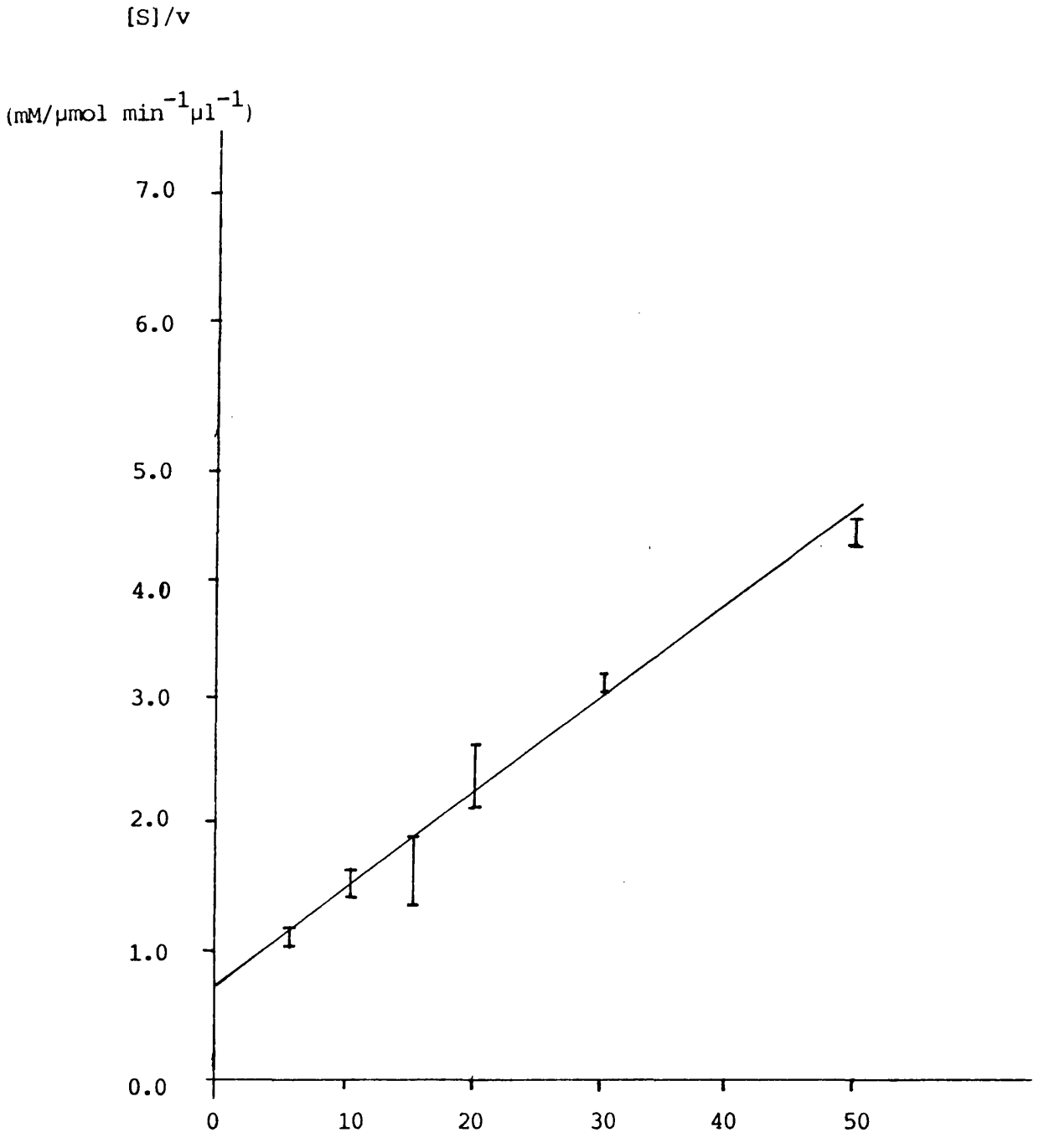
The Km of glyceraldehyde reductase from Sulfolobus acidocaldarius for NADPH was determined in an assay described in 2.2.5.14 with 50mM DL-glyceraldehyde. Error bars are SEM.

Graph 4.2.4. Showing the Half Reciprocal Plot for Glyceraldehyde Reductase From Sulfolobus acidocaldarus .



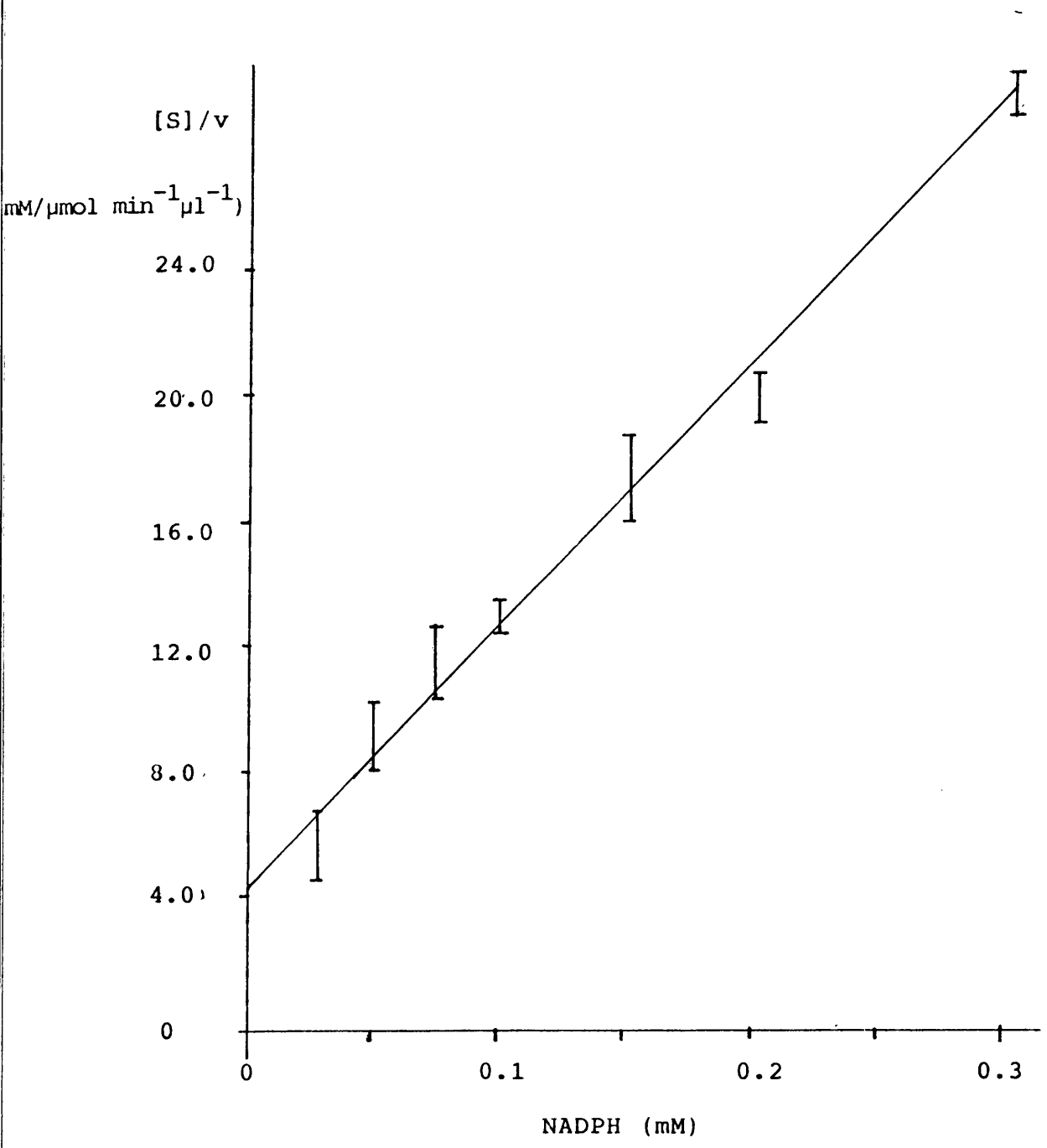
The Km of glyceraldehyde reductase from Sulfolobus acidocaldarius for DL-glyceraldehyde was determined in an assay described in 2.2.5.14 with 0.2mM NADPH . Error bars are SEM.

Graph 4.2.5. Showing The Half Reciprocal Plot For Glyceraldehyde Reductase From Thermoplasma acidophilum.



The Km of glyceraldehyde reductase from Thermoplasma acidophilum for DL-glceraldehyde was determined in an assay described in 2.2.5.14 with 0.2mM NADPH. Error bars are SEM.

Graph 4.2.6. Showing The Half Reciprocal Plot For Glyceraldehyde Reductase From Thermoplasma acidophilum.



The Km of glyceraldehyde reductase from Thermoplasma acidophilum for NADPH was determined in an assay described in 2.2.5.14 with 50mM DL-glyceraldehyde. Error bars are SEM.

organisms with a combination of substrates, but no activity was recorded. The following substrates were used: dihydroxyacetone phosphate with NADPH or NADH and DL-glycerol phosphate or (sn) glycerol 3-phosphate with NAD^+ or $NADP^+$. Commercially purified (sn)-glycerol 3-phosphate dehydrogenase was used to test all the assays containing NAD^+ and NADH.

c) Glyceraldehyde kinase (triokinase EC 2.7.1.28) and glycerol kinase (EC 2.7.1.30.).

D-glyceraldehyde + ATP $\longrightarrow$ D-glyceraldehyde 3-phosphate + ADP

glycerol + ATP $\longrightarrow$ (sn)-glycerol 3-phosphate + ADP

Glyceraldehyde kinase was assayed by following either the formation of ADP or D-glyceraldehyde 3-phosphate using DL-glyceraldehyde as the substrate. No activity was recorded in either organism. Glycerol kinase was assayed by following either the formation of ADP or (sn)-glycerol-3-phosphate and again no activity was recorded.

d) Triose phosphate isomerase

glyceraldehyde 3-phosphate $\longleftrightarrow$ dihydroxyacetone phosphate

This enzymic activity was found in both cell extracts with specific activities of 0.3 $\mu molemin^{-1}mg^{-1}$ for Tp. acidophilum and 0.052 $\mu molemin^{-1}mg^{-1}$ for S. acidocaldarius.

e) Glycerol dehydrogenase [EC 1.1.1.6.]

dihydroxyacetone + NAD(P)H $+H^+ \longleftrightarrow$ glycerol + $NAD(P)^+$

Assays were carried out using both cofactors on both organisms but no activity was recorded with either of the cell extracts.

4.3. Discussion.

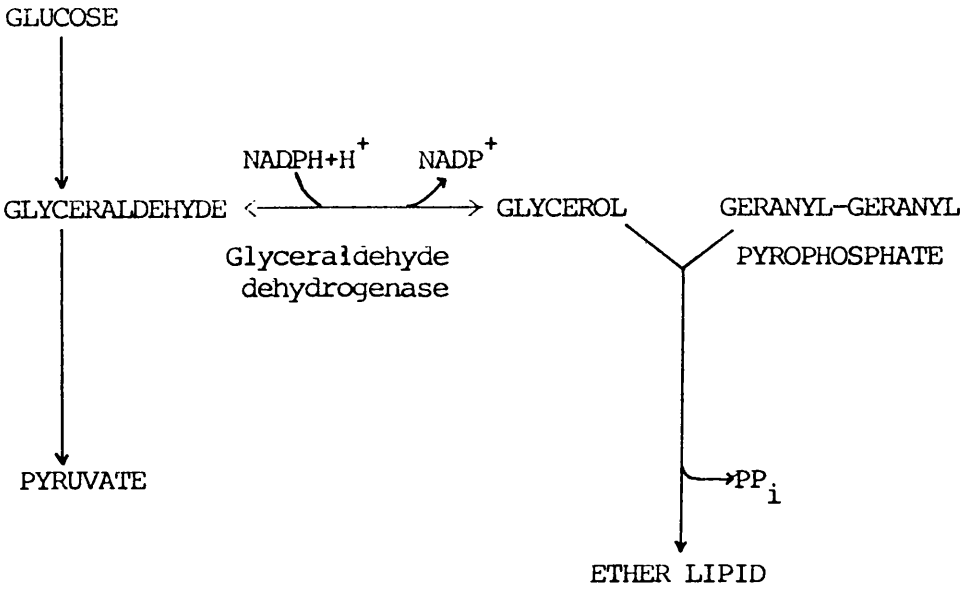
Both Tp, acidophilum and S. acidocaldarius possess a glyceraldehyde reductase activity catalysing the formation of glycerol from glyceraldehyde using NADPH as the reductant. The activities of glycerol kinase,glyceraldhyde kinase and DL-glycerol phosphate dehydrogenase could not be detected, even when the assay had the capacity to show a reaction with (sn)-glycerol 3-phosphate or DL-glycerol phosphate which includes (sn)-glycerol 1-phosphate.

DeRosa etal. (1982, 1986) have shown that in S.acidocaldarius glycerol is incorporated into the ether lipids with high efficiency and without change in hydrocarbon skeleton thereby discounting a keto, but not a phosphorylated, intermediate. Since no glycerol kinase could be detected, the observation would suggest that glycerol is formed from glyceraldehyde by the action of glyceraldehyde reductase, in both organisms. Thus in S. acidocaldarius glycerol synthesised from glyceraldehyde is directly incorporated into lipid. Energetically De Rosa et al. (1986) consider that the alkylation of glycerol by the intermediate geranyl geranyl pyrophosphate would present no conceptual difficultes. If so, the stereochemical form of the glycerol in the lipids must be provided by the alkylation. Figure 4.3.1 illustrates this possible pathway.

Tp. acidophilum possess similar enzymatic activities and ether lipids as those found in S, acidocaldarius. This may suggest that they share a common pathway. Glycerol labelling studies are required to confirm this.

Figure 4.3.1.Proposed pathway of lipid synthesis by Tp.acidophilum S.acidocaldarius.

The scheme outlines the possible pathway of ether lipid synthesis in the archaebacteria Tp. acidophilum and S. acidocaldarius.



In another archaebacterium, Halobacterium cutirubrum Kates and coworkers (Kates et al., 1970, Kates & Kushwaha, 1978) showed retention of ^{3}H in positions 1 and 3 of glycerol when cells were grown on glycerol. The ^{3}H on position 2 of glycerol was almost completely lost, suggesting it is metabolised to dihydroxyacetone. Wassef et al. (1970) have shown that H. cutirubrum possesses glycerol phosphate dehydrogenase whereas Baxter & Gibbons (1954) report a glycerol dehydrogenase activity. The action of only this latter enzyme on the radiolabelled glycerol will produce the observed labelling pattern, but if the substrate were phosphorylated the combined activity of the glycerol phosphate dehydrogenase and triose phosphate isomerase would result in loss of ^{3}H from C-1 as well as C-2. These results suggest glycerol is alkylated to form the lipids only via dihydroxyacetone.

In conclusion, in S. acidocaldarius, H. cutirubrum and perhaps Tp. acidophilum glycerol(not glycerol phosphate) is involved in ether lipid synthesis. This is fundamentally different from the pathway found in eukaryotes and eubacteria and may possibly reflect an early divergence of these organisms.

CHAPTER 5.

RADIORESPIROMETERIC ANALYSIS OF GLUCOSE CATABOLISM AND THE SYNTHESIS OF ACETATE.

5.1. Introduction

The aim of the research described in this chapter was to answer two questions arising from the proposed pathway (figure 3.3.1) of glucose catabolism in Tp. acidophilum, These are a) what reactions lead to a net synthesis of ATP and b) does the pathway operate to a significant extent in living cells?

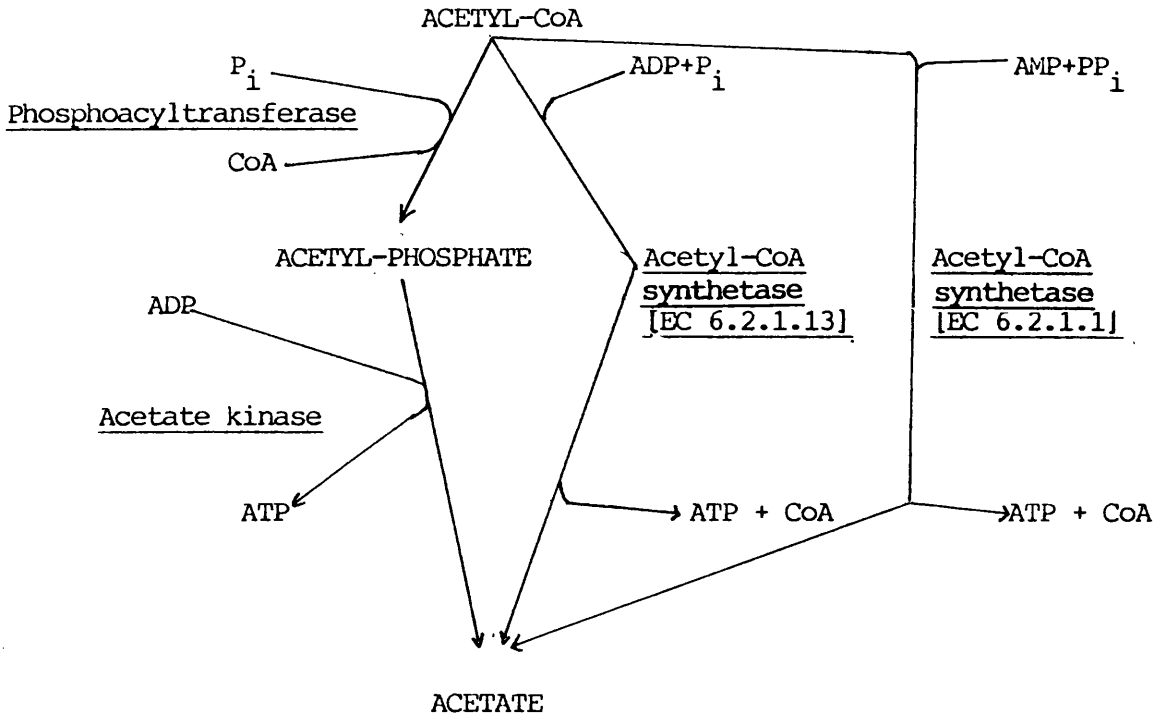
To investigate the synthesis of ATP, additional enzymatic activities of cell extracts of Tp. acidophilum were sought. Searcy & Whatley (1984) observed that cultures of Tp. acidophilum produced, and excreted in significant amounts, acetic acid when grown on glucose. The synthesis of acetic acid, combined with the possible synthesis of ATP to complete a fermentative pathway, was investigated

A plausible intermediate in the synthesis of acetate is acetyl-CoA which is known to be generated from pyruvate by the enzyme pyruvate:ferredoxin oxidoreductase (Kerscher et al., 1982). The enzymes summarised in figure 5.1.1 are those known to catalyze the conversion of acetyl-CoA to acetate. A search for these enzymes in cell extracts of Tp. acidophilum was carried out.

Also in this chapter, work is described which was designed to confirm or disprove the operation of the proposed non phosphorylated Entner-Doudoroff pathway in growing cells of Tp. acidophilum. To achieve this,

Figure 5.1.1. Possible Routes of Biosynthesis of Actate from Acetyl-CoA.

Three routes of synthesis of acetate from acetylCoA are known: a) indirect synthesis via the intermediate acetyl-phosphate, reactions are catalysed by the enzymes phosphoacyltransferase and acetate kinase b) direct synthesis by the catalysis of acetyl-CoA synthetase (ADP dependant and c) direct synthesis by the action of acetyl-CoA synthetase (AMP dependant).



radiorespirometeric analysis was used. This method has been used to show the catabolic pathways operating in a number of organisms, including Bacillus subtilis (Wang & Krackov 1962, Xanthomonas sp. (Zagallo & Wang 1967), Thiobacillus A2 (Wood et al., 1977) and Sulfolobus sp. (Wood et al.,1987). In each case, the operation of a combination of Embden-Meyerhof, Entner-Doudoroff and pentose phosphate pathways could be deduced. Radiorespirometeric analysis involves growth on specifically labelled D-^{14}C-glucoses and monitoring the rate of release of $^{14}CO_2$ from each. This approach was used with Tp.acidophilum.

In addition to the analysis of $^{14}CO_2$ evolution,the labelling pattern of the excreted ^{14}C-acetate was used independently to determine the active pathway. The fate of each carbon in glucose is dependant on the catabolic pathway utilized; thus the acetate will contain particular carbons derived from glucose. The ones retained are characteristic of the pathway of catabolism. The origin of the carbons in acetate was identified and used to show whether an Entner-Doudoroff pathway or the Embden-Meyerhof pathway was operating.

5.2. Results.

5.2.1. Synthesis of acetate.

5.2.1.1. Identification of acetate as an excretory product.

Acetate was identified both enzymatically in a coupled assay containing *E. coli* acetate kinase and chromatographically on Whatman $N^{o}3$ chromatography paper. The chromatographic identification was an essential feature of the investigation of the labelling patterns. All methods used are described in chapter 2.2.

To study the excretion of acetate, the formation of this acid was monitored in growing cultures of *Tp. acidophilum*. The results are summarised in figure 5.2.1.1. The production of acetic acid seems to lag behind the growth of the cells, suggesting that excretion occurs in the high cell density phase of late logarithmic and stationary cultures. The consumption of glucose was also followed and it was found that 40(±13)% of the total 50mM available glucose was lost from the media after completion of the fermentation. Thus 11.0(±2.6)% of the glucose lost from the media is oxidised to acetate, assuming 2 molecules of acetate produced per molecule of glucose.

5.2.1.2. Enzymatic activities.

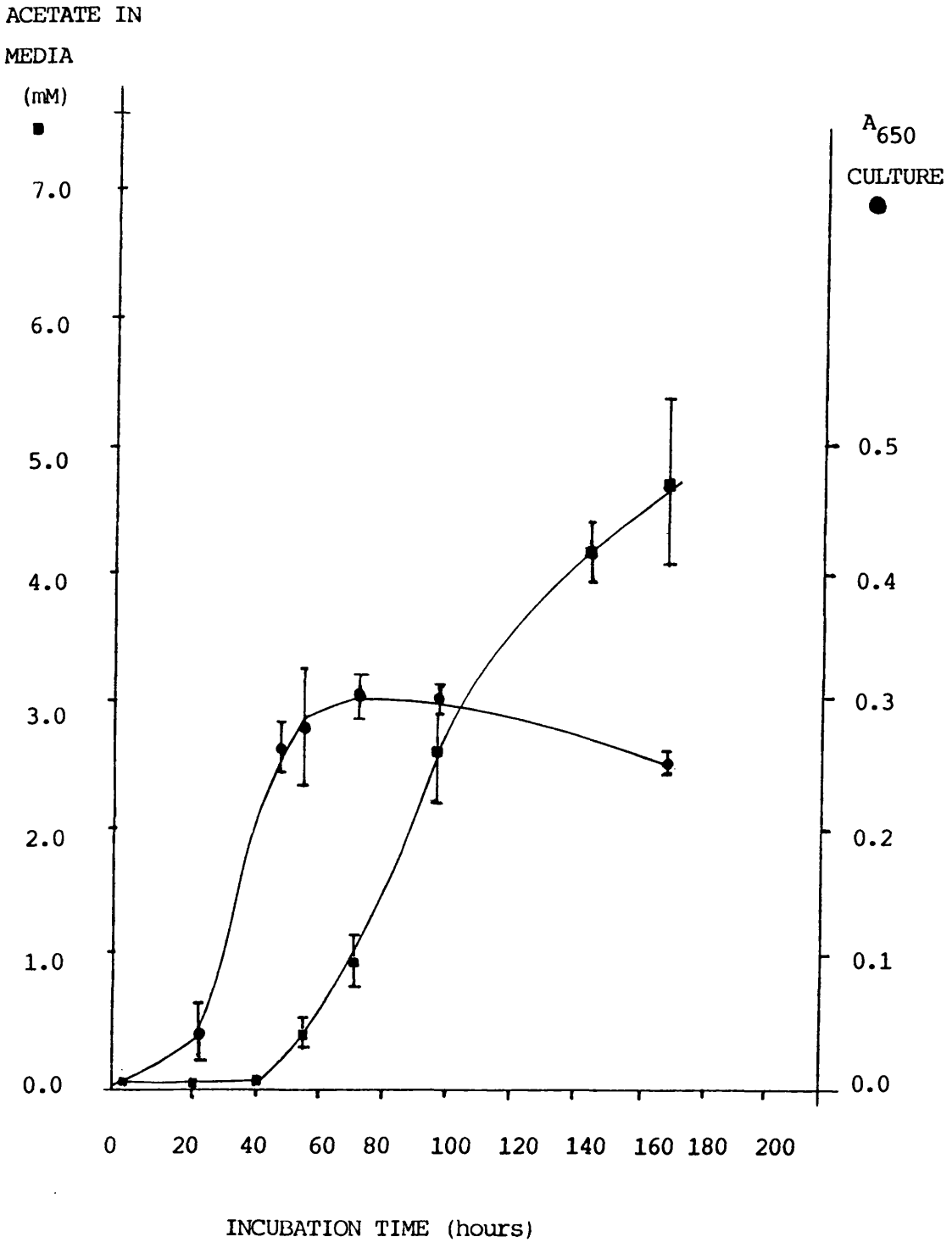
The enzymes involved in the synthesis of acetate from acetyl-CoA are summarised in figure 5.1.1. Assays were carried out with cell extracts of *Tp. acidophilum*.

Acetyl-CoA synthetase [EC 6.2.1.13.]

$$\text{acetyl-CoA} + \text{ADP} + P_i \longleftrightarrow \text{acetate} + \text{ATP} + \text{CoA}$$

Graph 5.2.1.1. Showing The Excretion Of Acetate And The Growth Of Thermoplasma acidophilum.

The concentration of acetate was determined as described in section 2.2.7. The error bars are the SEM of 4 experiments.



This enzyme was found to be active in cell extracts and possessed the following Km values, calculated from the direct linear plots (Eisenthal & Cornish-Bowden 1974) of the data obtained.

SUBSTRATES	Km VALUE
Acetate	2.93(±0.49) mM
ATP	0.15(±0.045)mM
Coenzyme A	0.0017(±0.0009)mM

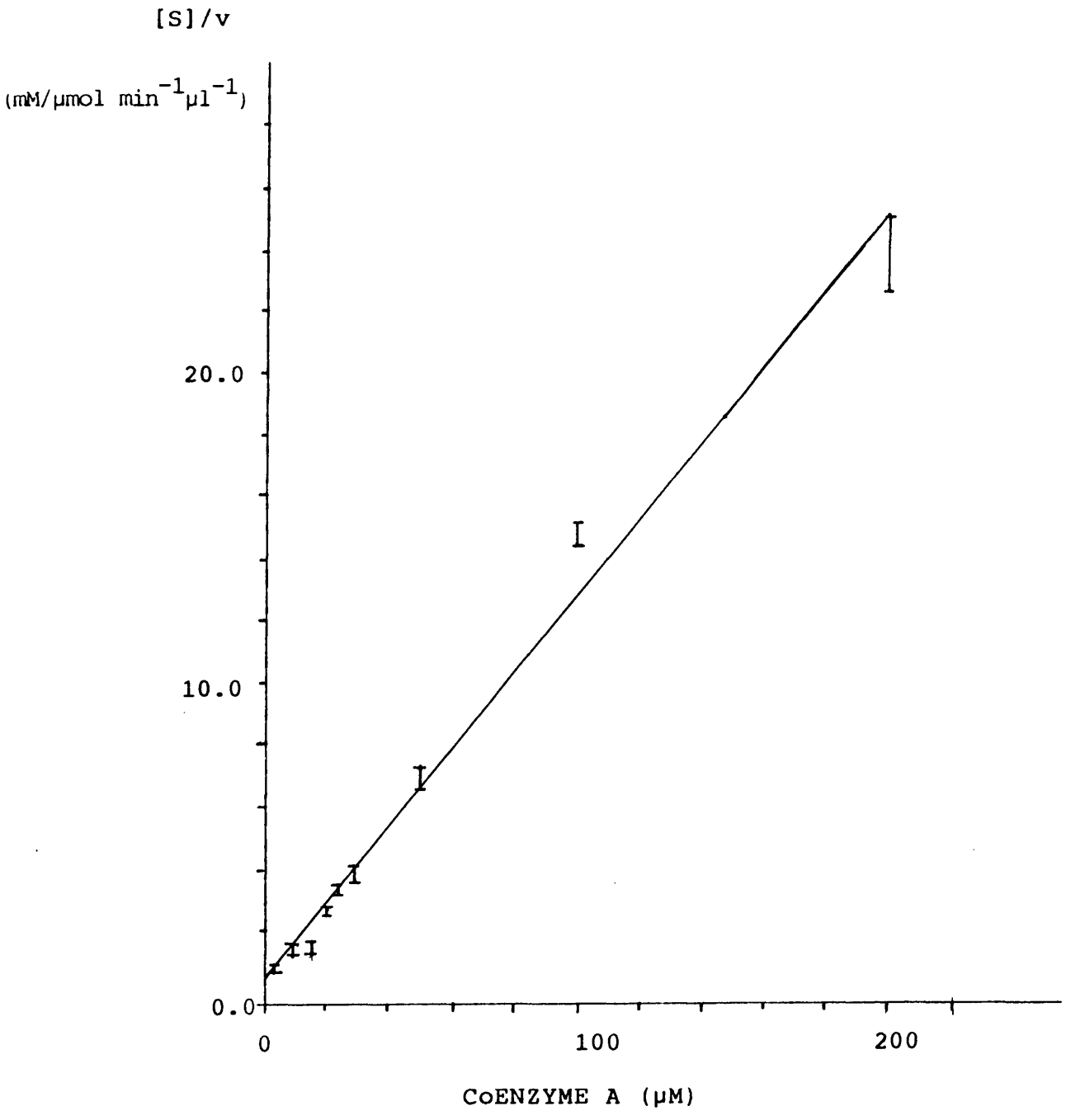
The enzyme was assayed for both the formation of acetyl-CoA and ADP. The specific activity for acetyl-CoA formation was 23.8(±1.5)nmole $min^{-1}mg^{-1}$ and for ADP was 22.8(±4.7)nmole $min^{-1}mg^{-1}$; hence for every molecule of acetyl-CoA formed 1 molecule of ADP is produced. The reverse reaction was also measured producing acetate from acetyl-CoA. The reaction was assayed as outlined in 2.2.5.2 by following the production of CoA using DTNB. The reaction was dependant on ADP,P_i and acetyl-CoA, and had a specific activity of 5.39(±0.06) nmole $min^{-1}mg^{-1}$.

Acetyl-CoA synthetase [EC 6.2.1.1.]

acetate + ATP + CoA $\longleftrightarrow$ AMP + PP_i + acetyl-CoA

No activity could be detected with AMP, pyrophosphate and acetyl-CoA, suggesting this widely distributed enzyme [EC 6.2.1.1.] is not present in Tp. acidophilum.

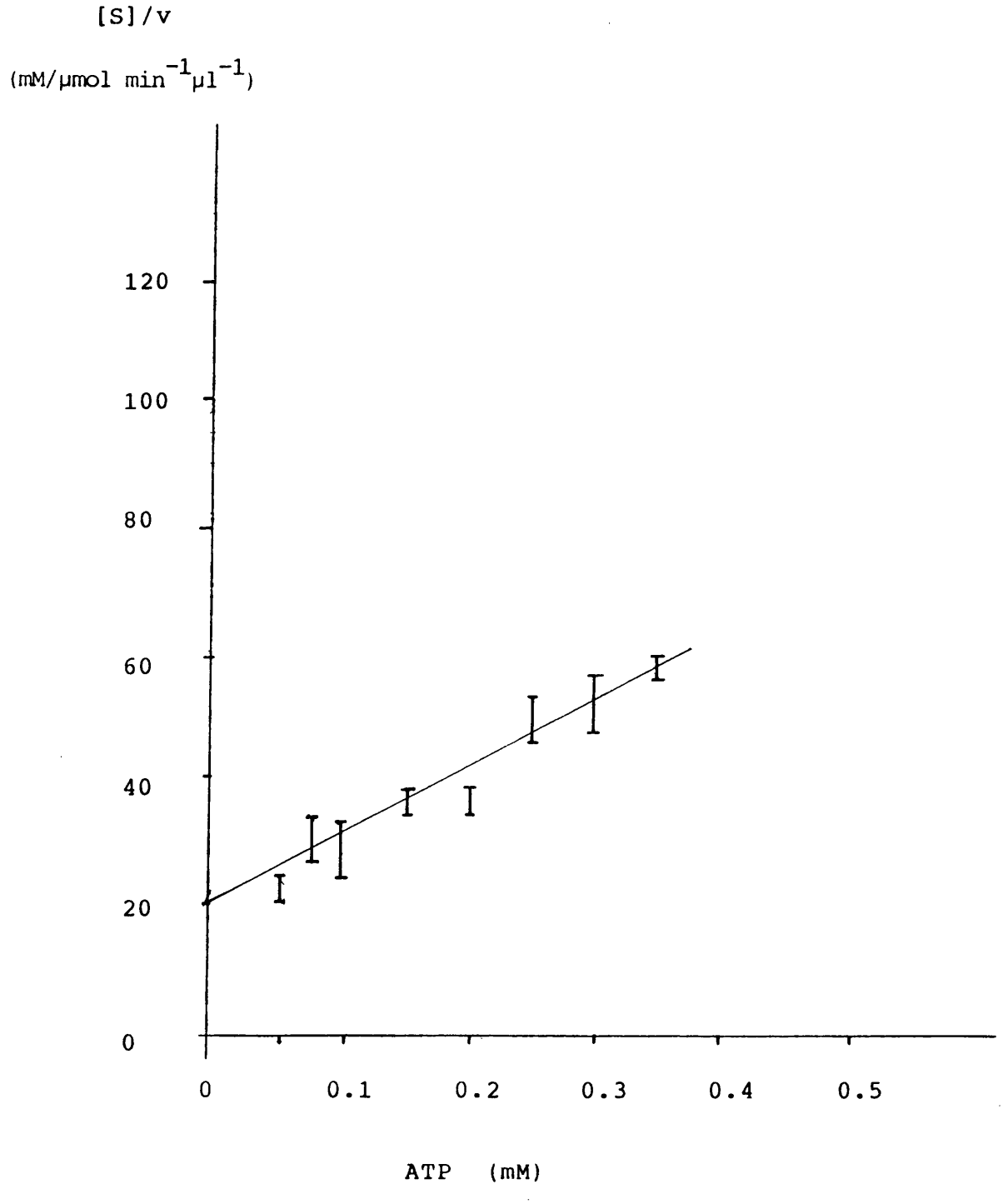
Graph 5.2.1.2. Showing The Half Reciprocal Plot For Acetyl-CoA Synthetase [EC 6.2.1.13.]



The Km of acetyl-CoA synthetase for Coenzyme A was determined in an assay described in 2.2.5.2. with 0.25mM ATP and 10mM sodium acetate. Error bars are SEM.

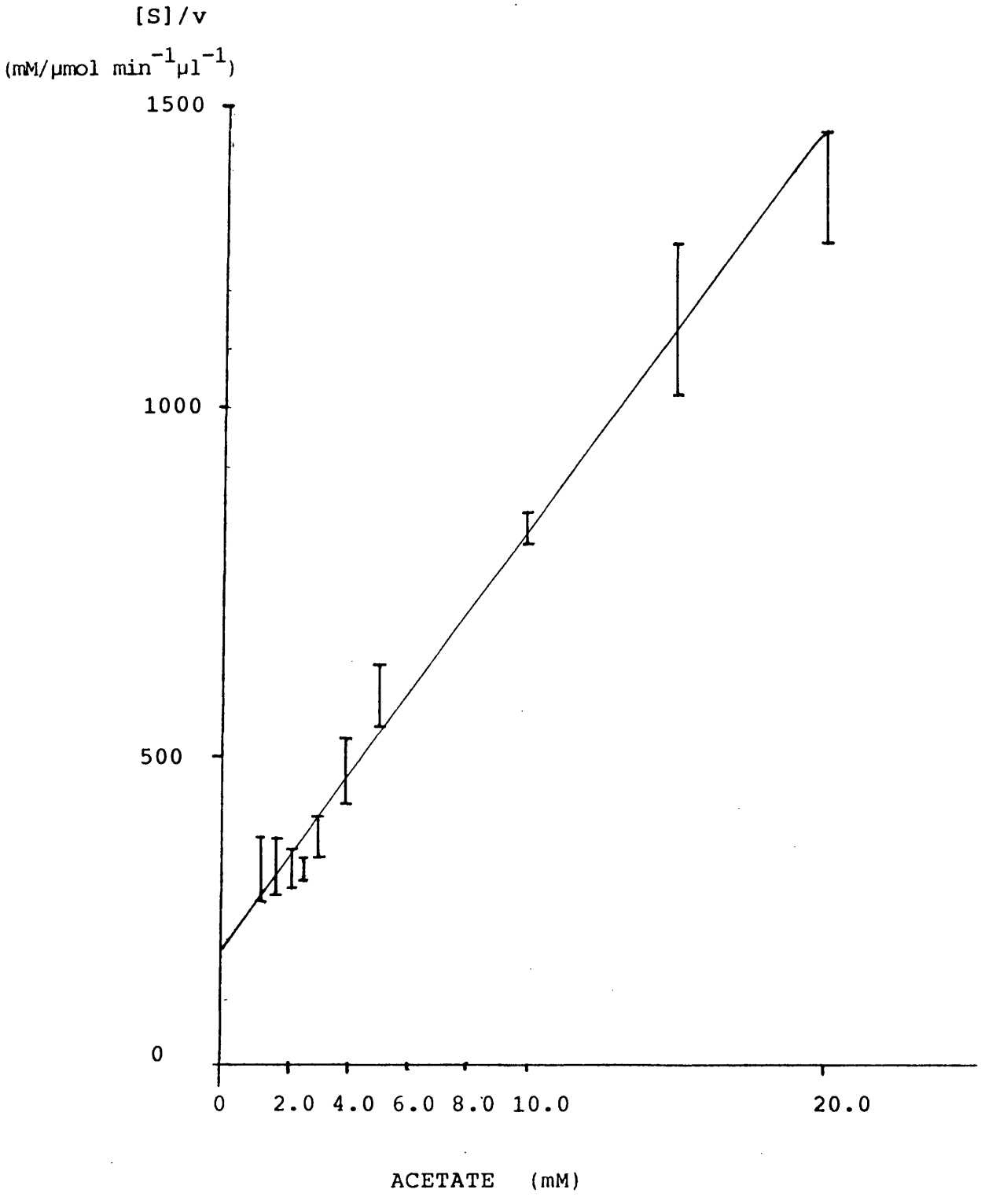
Graph 5.2.1.3. Showing The Half Reciprocal Plot For Acetyl-CoA Synthetase [EC 6.2.1.13.]

The Km of acetyl-CoA synthetase for acetate was determined in an assay described in 2.2.5.2. with 0.25mM ATP and 0.14mM CoA. Error bars are SEM.



Graph 5.2.1.4. Showing The Half Reciprocal Plot For Acetyl-Synthetase [EC 6.2.1.13.]

The Km of acetyl-CoA synthetase for ATP was determined in an assay described in 2.2.5.2. with 10mM sodium acetate and 0.14mM CoA. Error bars are SEMs.



Acetate kinase [EC 2.7.2.1].

acetate + ATP $\longrightarrow$ acetylphosphate + ADP

No activity could be recorded with this enzyme using an assay which monitored the ADP production from ATP and acetate.

Phosphotransacetylase [EC 2.3.1.8.].

acetyl-phosphate + CoA $\longleftrightarrow$ acetyl-CoA + P_i

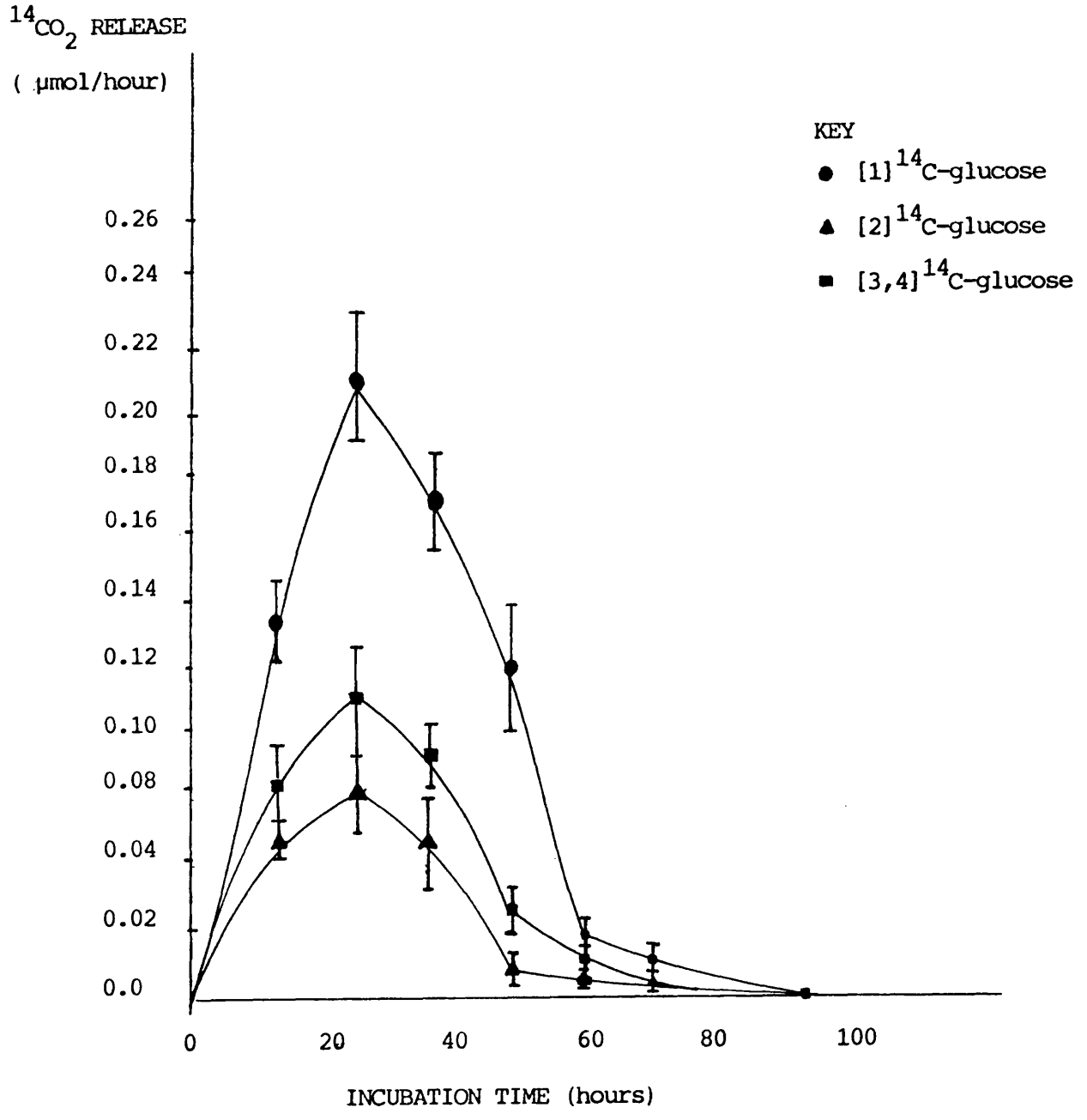
The enzyme was assayed in both directions by following the production of CoA with DTNB from acetyl-CoA and P_i and following acetyl-CoA formation from acetyl-phosphate and CoA. Neither of these approaches detected phosphotransacetylase activity in the extracts of Tp. acidophilum.

In all the assays described commercially purified enzymes were used to verify the method.

5.2.2. Radiorespirometeric analysis.

The release of $^{14}CO_2$ from [1],[2], and [3,4] ^{14}C-glucoses was monitored in growing cultures of Tp. acidophilum. The rate of release of $^{14}CO_2$ during incubation with the various isotopes is shown in figure 5.2.2.1. A peak of release of $^{14}CO_2$ occurred at approximately 25h under the conditions outlined in the methods section (2.2.8). The maximum rate of release with each isotope were calculated and expressed as a fraction of the rates and total obtained with [1]-^{14}C-glucose. The findings are given in figure 5.2.2.2. This data were compiled from 7 separate experiments. It must be noted that the specific radioactivity was the same in all treatments; thus [3,4]-^{14}C-glucose contained

Graph 5.2.2.1 Showing The Radiorespirometeric Analysis.



Error bars are the SEM of 7 experiments.

Table 5.2.2.2. Relative $^{14}CO_2$ release from Tp. acidophilum grown on D-[1], [2] and [3,4]-^{14}C-glucoses.

The following are the relative rates of release of $^{14}CO_2$ release from each ^{14}C-glucose at the peak of release. Each label is compared with the [1]-^{14}C-glucose peak release rate.

ISOTOPE	RELATIVE PEAK OF $^{14}CO_2$ RELEASE
[1]-^{14}C-GLUCOSE	100%
[2]-^{14}C-GLUCOSE	32.0(±7.0)%
[3,4]-^{14}C-GLUCOSE	44.9(±5.1)%

The following are the relative totals of $^{14}CO_2$ released over the complete fermentation with each ^{14}C-glucose. Each label is compared with the [1]-^{14}C-glucose total accummulated release.

ISOTOPE	RELATIVE TOTAL $^{14}CO_2$ RELEASE
[1]-^{14}C-GLUCOSE	100%
[2]-^{14}C-GLUCOSE	44.4(±3.8)%
[3,4]-^{14}C-GLUCOSE	57.3(±9.5)%

Errors are the SEM of 7 separate experiments.

50% [3]-^{14}C-glucose and 50% [4]-^{14}C-glucose, the two individual molecules having half the specific activity of the other isotopes.

5.2.3. Labelling pattern of excreted acetate.

Cultures of Tp. acidophilum which were grown with [1], [2], and [3,4] ^{14}C-glucose were analysed for the excretion of ^{14}C-labelled acetate, as given in the methods section (2.2.9). Since growth on [2]-^{14}C-glucose will result in the labelling of acetate regardless of whether the Enter-Doudoroff or the Embden-Meyerhof pathway is operating, the results are expressed as a fraction of the ^{14}C-acetate recovered with this isotope. It is however theoretically possible that no [2]-^{14}C-glucose appears in acetate if the pentose (phosphate) pathway is operating in a totally exhaustive cyclic mode such that all carbon in position [2] of glucose is expelled as CO_2 and only carbons in positions 4,5 and 6 continue to acetate via glycolysis.

^{14}C-labelled acetic acid was determined in a number of fermentations with Tp. acidophilum and each isotope, the relative amounts recovered are given in table 5.2.3.1.

The data, which were obtained from 7 separate experiments, suggest that very little of carbon 1 of glucose appears in either of the carbons of acetate; however carbons 3 and 4 of glucose, when combined, do provide carbon for acetate. When compared to that provided from carbon 2, only half as much is supplied by 3 and 4 . The significance of these results is discussed in the next section.

Table 5.2.3.1. The relative amounts of ^{14}C-acetate excreted by Tp. acidophilum when grown on [1],[2],[3,4]-^{14}C-glucose.

The following are the relative amounts of ^{14}C-acetic acid excreted during growth of Tp. acidophilum on [1], [2], and [3,4]-^{14}C-glucoses as recovered by solvent extraction and paper chromatography. The results are expressed as a percentage of the ^{14}C-acetate recovered after growth on [2]-^{14}C-glucose.

ISOTOPE	RELATIVE ^{14}C-ACETATE EXCRETED
[1]-^{14}C-GLUCOSE	8.4(±3.6)%
[2]-^{14}C-GLUCOSE	100%
[3,4]-^{14}C-GLUCOSE	44.4(±5.7)%

Errors are the SEM of 7 separate experiments.

5.3. Discussion.

5.3.1. Synthesis of acetate and ATP.

The production of acetate by Tp. acidophilum appeared to take place during late logarithmic to stationary phases, suggesting that a fermentation may be the result of oxygen limitation due to the high cell density of the culture. This is supported by the observation showing oxygen limitation in growing cultures of Tp. acidophilum only during late logarithmic to stationary phases (L.D. Smith, unpublished data).

Searcy & Whatley (1984) showed, by use of U-^{14}C-glucose that 15% of glucose consumed produces acetate (assuming that the glucose is uniformly labelled). This agrees with the figure of 11% found in this study.

Acetyl-CoA synthetase [EC 6.2.1.13.], found in Tp. acidophilum cell extracts, provides both acetate and ATP synthesis from acetyl-CoA and ADP. Thus the net reaction of the fermentation involving the non-phosphorylated Entner-Doudoroff pathway will be;

$$glucose + 2ADP + 2NADP^{+} + 2P_{I} \longrightarrow 2acetate + 2ATP + 2CO_{2} + 2NADPH + 2H_{2}O$$

This results in 2molecules of ATP produced per glucose oxidised to acetate, compared with a possible 4 ATPs via the Embden-Meyerhof pathway and a possible 3 for the conventional Entner-Doudoroff pathway.

The enzyme discovered is unusual in that it is ADP-dependant. The only other organism known to possess this enzyme is the eukaryotic parasite Entamoeba histolytica, where it is suggested that the conversion of acetyl-CoA to acetate is an important energy conservation step (Reeves et al.,1977). This maybe similar to the situation found

with Tp. acidophilum. The commonly found enzyme is the AMP utilising acetyl-CoA synthetase [EC 6.2.1.1.] which is present in both eubacterial and eukaryotes (Londesborough & Webster, 1974), and in the archaebacteria, Methanobacterium thermoautotrophicum (Oberlies et al.,1980) and Thermoproteus neutrophilus (Schafer et al.,1986). In these archaebacteria it is thought to have an acetate-assimilatory role. Thus, adoption of either enzyme appears to depend on its metabolic role, whether assimilatory or energy conserving. The reason may be due to the different equilibrium positions of the reactions, the reaction involving ADP will favour acetate formation in contrast to the reaction involving AMP and pyrophosphate.

Searcy & Whatley (1984) noted that acetic acid was toxic to Tp. acidophilum, a concentration of 3.0mM leading to a 50% reduction in the rate of growth. The reason for the toxicity of the acetate may be the result of the inhibition or even reversal of the reaction catalysed by acetyl-CoA synthetase, simply on the basis of mass action. This effect may be more pronounced than expected since, as Searcy & Whately (1984) pointed out, the acetic acid excreted will not be ionised at pH2.0 in the medium; hence it will be able to diffuse into the cells where it will be then ionised (internal pH6.0) and hence trapped. This will result in the accumulation of acetate, the product of the reaction, and could cause inhibition of acetyl-CoA synthetase.

5.3.2. Radiorespirometeric analysis.

Before considering the results of the radiorespirometeric analysis, it is pertinent to outline the basis of this approach. CO_2 released during the metabolism of glucose can be attributed to decarboxylating reactions catalysed by pyruvate decarboxylase and phosphogluconate dehydrogenase. The fate of each carbon atom in glucose is dictated by the pathway through which it is metabolised.Figure5.3.2.1 shows the labelling pattern and the origins of the carbons in the evolved CO_2 via the Embden-Meyerhof, Entner-Doudoroff or pentose phosphate pathways. It can be seen from this figure that the release of carbons 3 and 4 of glucose as CO_2 before carbon 1 is characteristic of the Embden-Meyerhof pathway; high [1] with 50% [3,4] (ie. low [3], Doudoroff pathway where C 1 is lost before C 3 and 4. The pentose phosphate pathway, however, will result in CO_2 containing carbons 1, 2 and 3 before carbons 4 of glucose. These theoretical predictions have been used and confirmed by a number of workers. The relative rates of CO_2-release obtained by other workers who were in possession of an extensive range of isotopes, enabled them to draw precise conclusions of the active pathway. Three sets of results are illustrated, taken from work published by Zagallo & Wang (1967), Wang & Krachov (1962) and Wood *et al.* (1987). in each case the operation of either one or more pathways of glucose catabolism was deduced from experiments using [1],[2],[3],[3,4] and [6]-^{14}C-glucoses; however, for simplicity, I have only quoted the rates and total release

Figure 5.3.2.1.The fate of the carbons of glucose resulting from its catabolism.

The fate of the carbons of glucose is illustrated in each of the different pathways

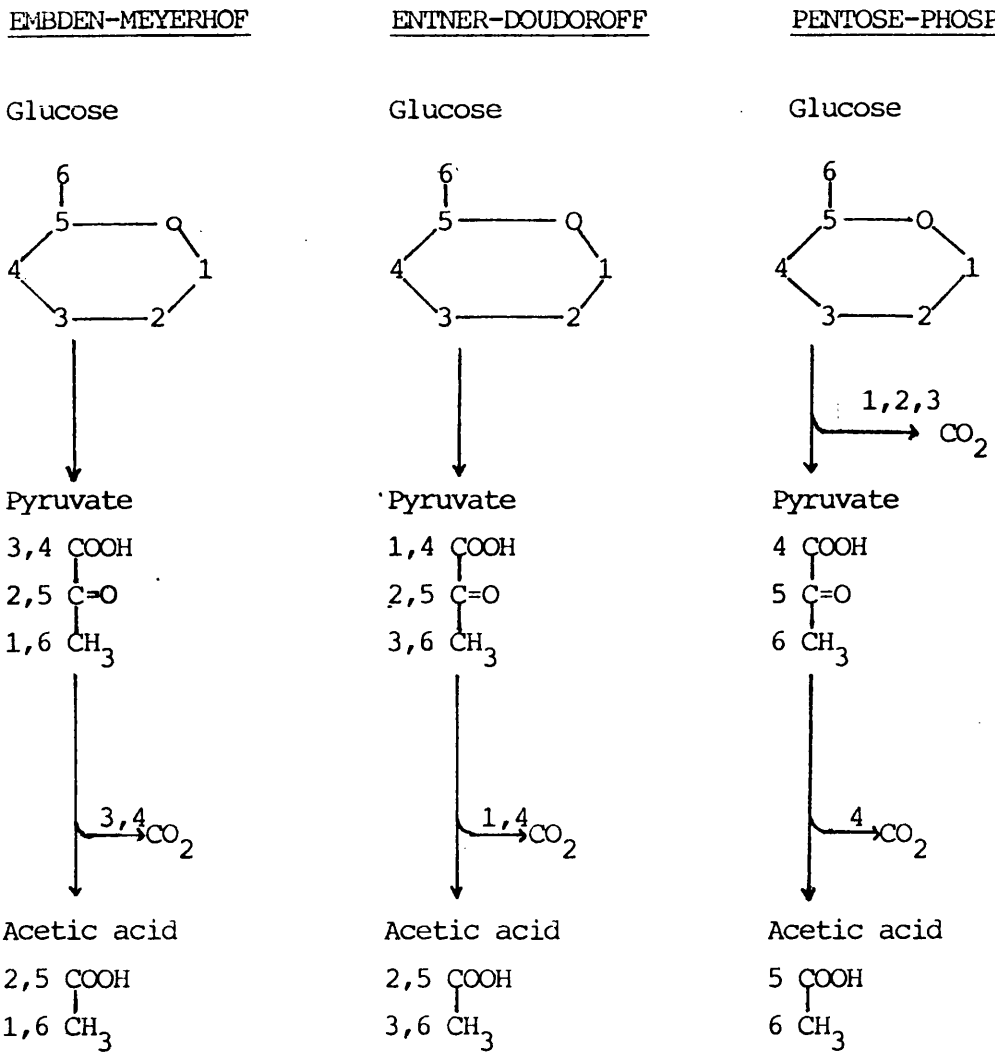


Table 5.3.2.2. Comparison of the Results of the Radiorespirometeric Analysis with other published Results.

The results of the radiorespirometeric analysis found with Tp. acidophilum are compared with results and conclusions drawn by other authors using other organisms.

ORGANISM	% OF PEAK RELEASE OF $^{14}CO_2$			CONCLUSIONS	REFERENCE
	[1]	[2]	[3,4]		
Tp.acidophilum	100	32.0(±7)	44.9(±5)	ENTNER DOUDOROFF & PENTOSE PHOS.	
Xanthomonas sp.	100	61.8(±4)	58.4(±5)	ENTNER-DOUDOROFF & PENTOSE PHOS.	1
Bacillus subtilis	100	75.0(±5)	213(±18)	EMBDEN-MEYERHOF & PENTOSE PHOS.	2

ORGANISM	% OF TOTAL RELEASE OF $^{14}CO_2$			CONCLUSIONS	REFERENCE
	[1]	[2]	[3,4]		
Tp.acidophilum	100	44.4(±3.8)	57.3(±9.5)	ENTNER-DOUDOROFF & PENTOSE PHOS.	
Xanthomonas sp.	100	68.8(±4.4)	69.8(±4.2)	ENTNER-DOUDOROFF & PENTOSE PHOS.	1
Bacillus subtilis	100	100	141	EMBDEN-MEYERHOF & PENTOSE PHOS.	2
S.brierleyi	100	10	50	ENTNER-DOUDOROFF	3

References; 1. Zagallo & Wang (1967) 2.Wang & Krachov (1961)
3. Wood et al. (1987)

Errors are the SEM of 7 separate experiments.

of $^{14}CO_2$ of isotopes used in analysing Tp. acidophilum. The conclusions drawn by each author are given for each organism (table 5.3.2.2.). The results are compared with those obtained with Tp. acidophilum and the conclusions are given. The conclusions are based on the following features of glucose carbons released as CO_2; high[3,4] exceeding [1] (i.e. over 100%) suggests the Embden-Meyerhof pathway; high [1] with 50% [3,4] (ie. low [3], high [4]) suggests the Entner-Doudoroff pathway; high [1] and [2] (approaching 100%) suggests a cyclic pentose phosphate pathway.

From both the peak $^{14}CO_2$ release rates and the $^{14}CO_2$ totally released during the growth of Tp. acidophilum with the various ^{14}C-glucoses, it can be concluded that the Entner-Doudoroff (and not the Embden-Meyerhof pathway) operates in this archaebacterium, with a contribution from a cyclic pentose phosphate pathway.

5.3.3. Acetate labelling.

To understand the results from this work, the theoretical basis of the experiments must be explained. Glucose is catabolised to 2 molecules of pyruvate, the distribution of the glucose carbons in the pyruvate depending on whether the sugar has been metabolised via the Entner-Doudoroff or the Embden-Meyerhof pathways (figure 5.3.2.1.). Pyruvate formed via the Embden-Meyerhof pathway is composed of carbons 1 and 6 forming the methyl, 2 and 5 the carbonyl, and 3 and 4 the carboxyl groups. However, if formed via the Entner-Doudoroff pathway, carbons 3 and 6 form the

Figure 5.3.3.1. Comparison of the predicted and the observed relative ^{14}C-acetate excreted by Tp. acidophilum.

The predicted amounts of ^{14}C- acetate, calculated from the known labelling patterns of each pathway, are compared with the observed aata. The theoretic basis of the labelling patterns is given in figure 5.3.2.1.

PATHWAY	RELATIVE AMOUNT OF ^{14}C-ACETATE EXCRETED WITH EACH ISOTOPE		
	^{14}C-GLUCOSE		
	[1]	[2]	[3,4]
ENTNER-DOUDOROFF	0%	100%	50%
EMBDEN-MEYERHOF	100%	100%	0%
PENTOSE-PHOSPHATE	0%	0%	0%
OBSERVED WITH Tp. acidophilum	8.4(±3.6)	100%	44.4(±5.7)

Errors are SEM of 7 experiments.

methyl, 2 and 5 the carbonyl, and 1 and 4 the carboxyl groups. Pyruvate is decarboxylated by pyruvate:ferredoxin oxidoreductase to form acetyl-CoA; thus metabolism via an Entner-Doudoroff pathway results in the loss of carbons 1 and 4 as CO_2 but, if via an Embden Meyerhof pathway, loss of 3 and 4 will result. Thus, tracing the metabolism through to acetate via acetyl-CoA synthetase, the expected labelling patterns for each molecule are as follows: via the Embden Meyerhof pathway, C 1 and 6 of glucose form the methyl and, C 2 and 5 form the carboxyl group. However, via the Entner-Doudoroff pathway, C 3 and 6 form the methyl but C 2 and 5 form the carboxyl.

In conclusion, if the Embden-Meyerhof pathway is operating, then Tp. acidophilum grown on [1]-^{14}C-glucose should excrete ^{14}C-labelled acetate with the same specific radioactivity as found with [2]-^{14}C-glucose; however,if the Entner-Doudoroff pathway is operating no ^{14}C-acetate will be excreted with [1]-^{14}C-glucose but with [3,4]-^{14}C-glucose half the comparative specific activity will appear in the acetate. If the pentose phosphate cycle was operating giving rise to trioses in the absence of fructose 1-6-phosphate aldolase, then no carbons from 1, 2 or 3 of glucose will reach pyruvate; hence, acetic acid will not be radiolabelled.

The results obtained are compared with the predicted labelling pattern in table 5.3.3.1. In this comparision of the results obtained with those theoretically predicted, the data show that the acetic acid excreted by Tp. acidophilum is a product of an Entner-Doudoroff pathway and not of

an Embden-Meyerhof pathway or an 'isolated' pentose phosphate pathway.

These results, and those found independently from the radiorespirometeric analysis together with the enzymology and product analysis described in chapter 4, provide strong evidence for the catabolism of glucose via the non-phosphorylated Entner-Doudoroff pathway in Tp. acidophilum.

CHAPTER 6.

CHARACTERISATION OF GLUCOSE DEHYDROGENASE FROM Thermoplasma acidophilum.

6.1. Introduction.

The purification and partial characterisation of glucose dehydrogenase [EC 1.1.1.47] from Tp. acidophilum are described in this chapter.

A number of dehydrogenases from archaebacterial species have been characterised. Danson(1988) has noted that dual cofactor specificity, that is, the ability to utilise both NAD^+ or $NADP^+$, is uncommon in eubacterial and eukaryotic dehydrogenases; this is in contrast to the dehydrogenases of thermophilic archaebacteria. The following archaebacterial dehydrogenases show activity with both NAD^+ and $NADP^+$: isocitrate dehydrogenase from Sulfolobus acidocaldarius (Danson & Wood, 1984), malate dehydrogenase from Methanobacterium hungatei (Sprott et al., 1979), Tp. acidophilum and S. acidocaldarius (Grossebuter et al.,1986), glutathione reductase from S. acidocaldarius (Smith et al., 1988) and glucose dehydrogenase from S. solfataricus (Giardina et al.,1986).

As presented in chapter 3, Tp. acidophilum cell extracts possessed both $NADP^+$ and NAD^+-dependant glucose dehydrogenase activities. It was of interest to investigate whether this is two separate enzymes or one enzyme with dual specificity. Evidence for a single protein is provided from kinetic analysis, from the purification of the

enzyme and from the nature of the substrate specificity with respect to cofactors as well as monosaccarides.

Glucose dehydrogenase has been isolated from a variety of eukaryotic and eubacterial sources including mammalian liver (Campbell et al., 1982), cyanobacteria (Pulich et al., 1976), Pseudomonas, Klebsiella, Serratia and other oxidative species (Matsushita et al., 1980) an alkalophilic Corynebacterium (Kobayashi & Horikoshi, 1980) and many members of the Bacillaceae (Vasantha et al., 1983). The enzyme has also been purified from the archaebacterium Sulfolobus solfataricus (Giardina et al., 1986).

Glucose dehydrogenase show a variety of catalytic properties, such as their ability to utilise either NAD^+, $NADP^+$ or both, and their specificity for a variety of hexoses. There is also a variety of oligomeric forms. The enzyme from Corynebacterium sp. is a single polypeptide of native Mr=55,000, it is active only with NAD^+ and oxidises only glucose and xylose (Kodayashi & Horikoshi, 1980). On the other hand, the enzyme from Gluconobacter sp. is $NADP^+$ dependant, is capable of oxidising only glucose and mannose and is a tetrameric protein of native Mr=150,000 (Avigad et al., 1968; Adachi et al., 1980). S.solfataricus glucose dehydrogenase was shown by Giardina et al., (1986) to be a tetrameric enzyme of native Mr=124,000, which was catalytically active both with NAD^+ and $NADP^+$. With NAD^+ as the coenzyme, D-glucose and D-idose were oxidised high rates,

whereas with $NADP^+$ a broader range of monosaccarides was oxidised but at a lower rate. The K_m^{app} for NAD^+ was 20 times lower than the K_m^{app} for $NADP^+$ with glucose. Also Mg^{2+} and Ca^{2+} ions activated the enzyme activity.

Another glucose dehydrogenase that possesses dual cofactor specificity is found in Bacillus sp. during sporulation. The tetrameric enzyme from B. subtilis has a native Mr=120,000 and exhibits a monomer-oligomer equilibrium. It can use both NAD^+ and $NADP^+$ with equal specificities to oxidise glucose but not mannose (Fujita et al.,1977). Similarly the enzyme from B.cereus is active with both cofactors (Sandoff,1966). Giardina et al. (1986) point out that the heat-stable Bacillus sp. enzyme formed during sporulation is similar to the archaebacterial glucose dehydrogenase with respect to catalytic and molecular properties.

In view of the diversity of structure and catalysis of other glucose dehydrogenases, it was of interest to characterise the enzyme from the thermophilic archaebacterium Tp. acidophilum.

6.2. Results

6.2.1. Substrate specificity.

6.2.1.1. Cofactors.

As described in chapter 4, both an $NADP^+$ and NAD^+ dependant glucose dehydrogenase activities were detected in unfractionated cell extracts of Tp. acidophilum. To investigate the possibility of a dual specific enzyme, a kinetic analysis of the glucose dehydrogenase was carried out as described by Danson & Wood(1984). Assays of the enzyme in crude extracts of Tp. acidophilum were carried out in $NADP^+$, NAD^+ and $NADP^+$ plus NAD^+. The rate of the combined assay was compared with that theoretically determined assuming a) it was due to the activity of two independent enzymes, ie. the simple addition of the rates found separately with each cofactor (equation 1) and b) assuming the activities were due to a single enzyme. In this latter case the cofactors will compete with each other for the oxidation of glucose, and will do so with Ki values equal to their Km values, assuming they bind at the same active site on a single enzyme. The theoretical enzyme activity for one enzyme capable of utilising both NAD^+ and $NADP^+$ is given by equation 2.

Since the Km for NAD^+ was too large to determine accurately, the term $[NAD]/Km^{NAD}$ was assumed to approach zero.

Table 6.2.1.1. shows the results of this analysis where the observed rates are compared with the theoretical rates calculated from the equations 1 and 2 given. The results show that the $NADP^+$ and NAD^+ linked enzymatic

Figure 6.2.1.1. Equations used in the kinetic analysis.

Equation 1; predicts the resultant rate of an assay containing NAD^+ and $NADP^+$, in which two enzymes, each capable of utilising only one cofactor, are present:

$$V_{total} = V_{NAD} + V_{NADP} \qquad [1]$$

Equation 2; predicts the resultant rate of an assay containing NAD^+ and $NADP^+$, in which a single enzyme which is capable of utilising both cofactors, is present:

$$V_{total} = \frac{V_{NAD}(1 + [NAD]/Km^{NAD}) + V_{NADP}(1 + [NADP]/Km^{NADP})}{1 + [NAD]/Km^{NAD} + [NADP]/Km^{NADP}} \qquad [2]$$

where; V_{total} is the predicted rate in the presence of $NADP^+$ and NAD^+.

V_{NAD} and V_{NADP} are the respective velocities of assays containing [NAD] only or [NADP] only.

The Km values for each cofactor are given in chapter 4.

Table 6.2.1.2. Kinetic analysis of cofactor specificity of Tp. acidophilum glucose dehydrogenase.

This table compares the observed rates of assay containing NAD^+, $NADP^+$ and a mixture of NAD^+ and $NADP^+$ with the theoretical rates assuming either a single dual specific enzyme or two single specific enzyme. Assays contained 50mM D-glucose.

ASSAY MIXTURE	OBSERVED ENZYME ACTIVITY $\mu mol.min^{-1}$	THEORETICAL ENZYME ACTIVITY $\mu mol.min^{-1}$	
		ONE ENZYME (EQUATION 2)	TWO ENZYMES (EQUATION 1)
0.2mM $NADP^+$	0.017(±0.0001)		
5.0mM NAD^+	0.0028(±0.0001)		
0.2mM $NADP^+$ + 5.0mM NAD^+	0.018(±0.001)	0.0177	0.020(±0.002)
0.10mM $NADP^+$	0.00826(±0.0003)		
5.0mM NAD^+	0.00174(±0.0007)		
0.10mM $NADP^+$ + 5.0mM NAD^+	0.00699(±0.0001)	0.00884	0.010(±0.0003)
0.05mM $NADP^+$	0.00108(±0.00001)		
5.0mM NAD^+	0.00048(±0.00001)		
0.05mM $NADP^+$ + 5.0mM NAD^+	0.00122(±0.0005)	0.00082	0.00157(±0.0001)

Table 6.2.1.3. Kinetic analysis of hexose specificity of Tp. acidophilum glucose dehydrogenase.

This table compares the observed rates of assay containing glucose galactose and a mixture of glucose and galactose with the theoretical rates assuming either a single dual specific enzyme or two single specific enzymes. Assays contained 0.2mM $NADP^+$ as the cofactor.

ASSAY MIXTURE	OBSERVED ENZYME ACTIVITY $\mu mol.min^{-1}$	THEORETICAL ENZYME ACTIVITY $\mu mol.min^{-1}$	
		ONE ENZYME (EQUATION 2)	TWO ENZYME (EQUATION 1)
10mM galactose	0.119(±0.01)		
10mM glucose	0.215(±0.001)		
10mM galactose 10mM glucose	0.212(±0.008)	0.222	0.334(±0.01)
5mM galactose	0.0755(±0.002)		
5mM glucose	0.142(±0.0023)		
5mM galctose 5mM glucose	0.155(±0.021)	0.163	0.218(±0.02)

activities were not additive, but that competition between the cofactors appeared to occur, suggesting that they might bind to the active site on a single glucose dehydrogenase.

6.2.1.2. Hexose.

As described in chapter 4, an NADP-dependant galactose dehydrogenase activity was found in unfractionated cell extracts of Tp. acidophilum. To investigate whether or not glucose dehydrogenase is the same enzyme species as galactose dehydrogenase, the same method of analysis was applied as that described for the investigation of cofactor specificity. Table 6.2.1.2 summarizes the competition of glucose and galactose for the reduction of $NADP^+$.

The data suggest that glucose and galactose compete for the same active site on a single enzyme species.

6.2.2. Purification of glucose dehydrogenase.

To provide further evidence that this enzyme possesses dual specificity with respect to cofactors and hexoses, and also to characterise its molecular properties, glucose dehydrogenase was purified. Table 6.2.2.1 shows a typical set of results of the purification of glucose dehydrogenase from approximately 1.5g of frozen cells of Tp. acidophilum. The method is described fully in the methods section.

A methanol fractionation proved a quick, convenient and valuable step in the purification of the enzyme from sonicated cell extracts. The thermostable enzyme was extremely stable in this solvent, being reversibly denatured in 90%(w/v) methanol and returning into solution in 30%(w/v) methanol with nigh efficiency and retention of activity.

Table 6.2.2.1. Purification table of Tp. acidophilum glucose dehydrogenase

STAGE	TOTAL PROTEIN (mg)	TOTAL ACTIVITY $\mu mol.min^{-1}$	SPECIFIC ACTIVITY $\mu mol.min^{-1}mg^{-1}$	YIELD (%)
Sonicated cells	300	23.7	0.079	100
Methanol fractionation 55%-30% (v/v)	4.8	18.7	3.89	80
Mono Q ion exchange 20mM triethanolamine pH7.5, eluted with 0.25M NaCl	1.8	12.7	7.1	54
Mono P chromatofocusing pH gradient pH7-4 eluted at pH4.3	0.14	9.54	68.1	40
Superose 12, 50mM phosphate buffer pH7.2, 0.15M NaCl	0.012	2.24	187	17*

* The final yield was calculated assuming all the preparation was purified to this stage.

Many proteins in the cell extract were irreversibly denatured by this treatment with methanol, whereas others were soluble in high concentrations of methanol (<55% (v/v). Thus the solvent fractionation procedure resulted in a considerable purification of glucose dehydrogenase. This stage provided a good substitute for the more conventional ammonium sulphate fractionation. The protein concentration in this and the first step of the purification was determined by the method described by Lowry et al. (1951).

The Pharmacia Fast Protein Liquid Chromatography (FPLC) system was successfully used in the last three stages of the purification as fully described in 2.2.11. The MonoQ ion exchange column with a NaCl gradient of 0-0.6M eluted the bound enzyme which was then applied to the Mono P chromatofocusing column and a pH gradient of 7.0-4.0 eluted the enzyme. The purified preparation was then applied to a Superose 12 gel filteration column equilibrated with 50mM phosphate buffer, pH7.2, and 0.15M NaCl. The protein concentration in fractions from each of these steps was determined spectrophotometerically as described by Warburg & Christian (1942).

The purity of the enzyme preparations was determined by slab SDS-polyacrylamide gel electrophoresis, after which proteins were visualised by the sensitive silver staining method of Neilson & Brown (1984).

6.2.3. Molecular properties.

The native molecular weight (Mr) of glucose dehydrogenase was determined by gel fitration using the FPLC Superose 12 column. The column, equilibrated with 50mM K^+phosphate buffer, pH7.2, containing 0,15M NaCl, was calibrated using rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Mr=144,000), pig heart malate dehydrogenase (Mr70000), yeast phosphoglycerate kinase (Mr=47,000) and rabbit muscle phosphoglycerate mutase.(Mr=57,000). The data were plotted as $\log_{10}$ Mr versus Kd(the distribution coefficient) as shown in figure 6.2.3.1.. The Kd determined under the same conditions for glucose dehydrogenase was 0.328±0.01 which corresponds to an Mr of 70,000(±2000) on the calibrated Superose 12 FPLC column.

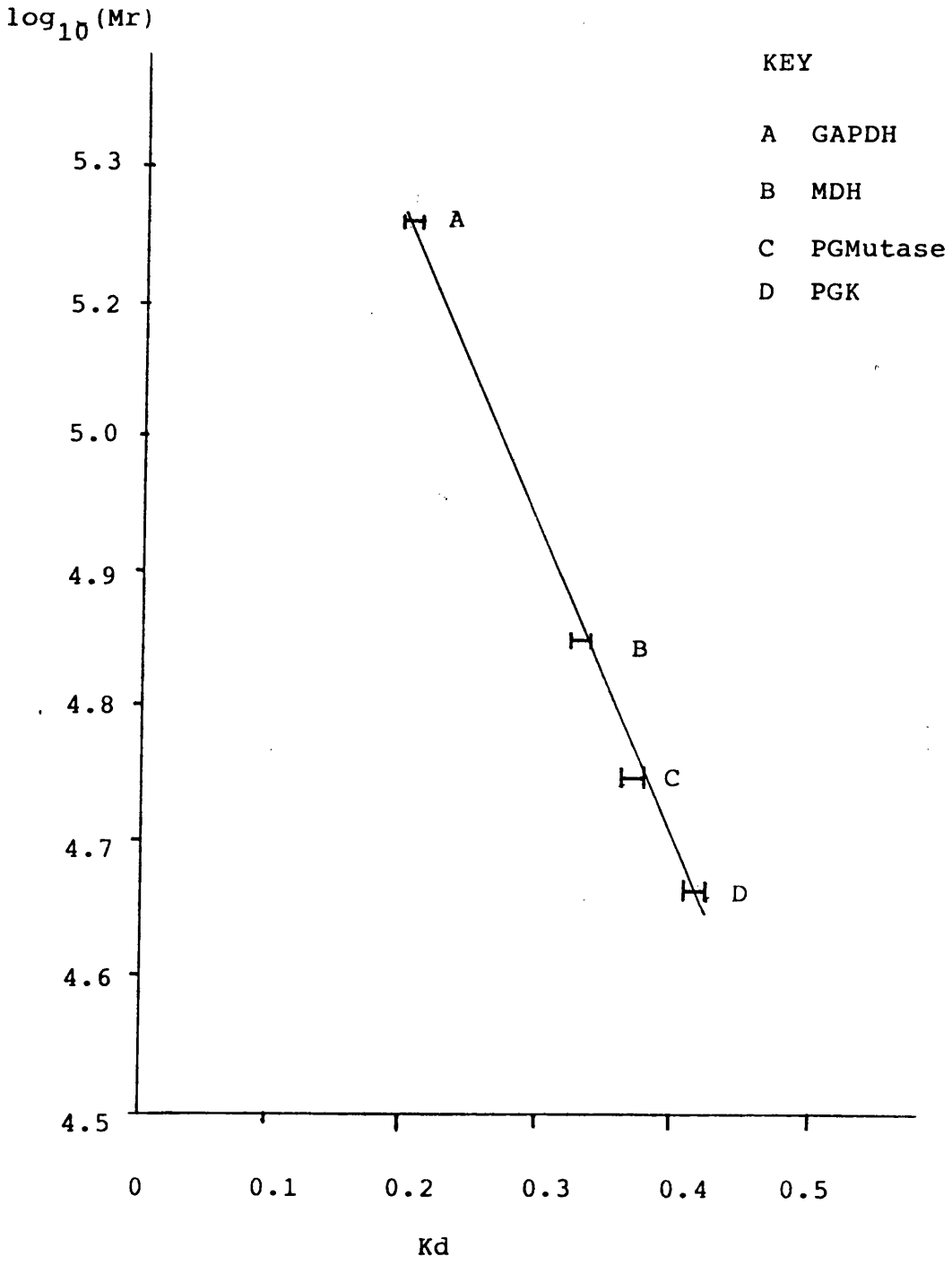
SDS polyacrylamide gel electrophoresis, calibration with bovine serum albumin (Mr=68,000), ovalbumin (Mr=45,000), GAPDH (Mr=44,000), carbonic anhydrase (Mr=29,000),trypsin inhibitor (Mr=20,100) and pig heart (PH) citrate synthase (Mr=49,000), was performed on the purified glucose dehydrogenase (figure 6.2.3.2.). The subunit molecular weight of the glucose dehydrogenase was calculated to be either 41,700 or 36,000 under the dissociating conditions, as two bands were found in the preparation.

This would suggest that Tp. acidophilum glucose dehydrogenase is a dimer of approximately Mr=70,000.

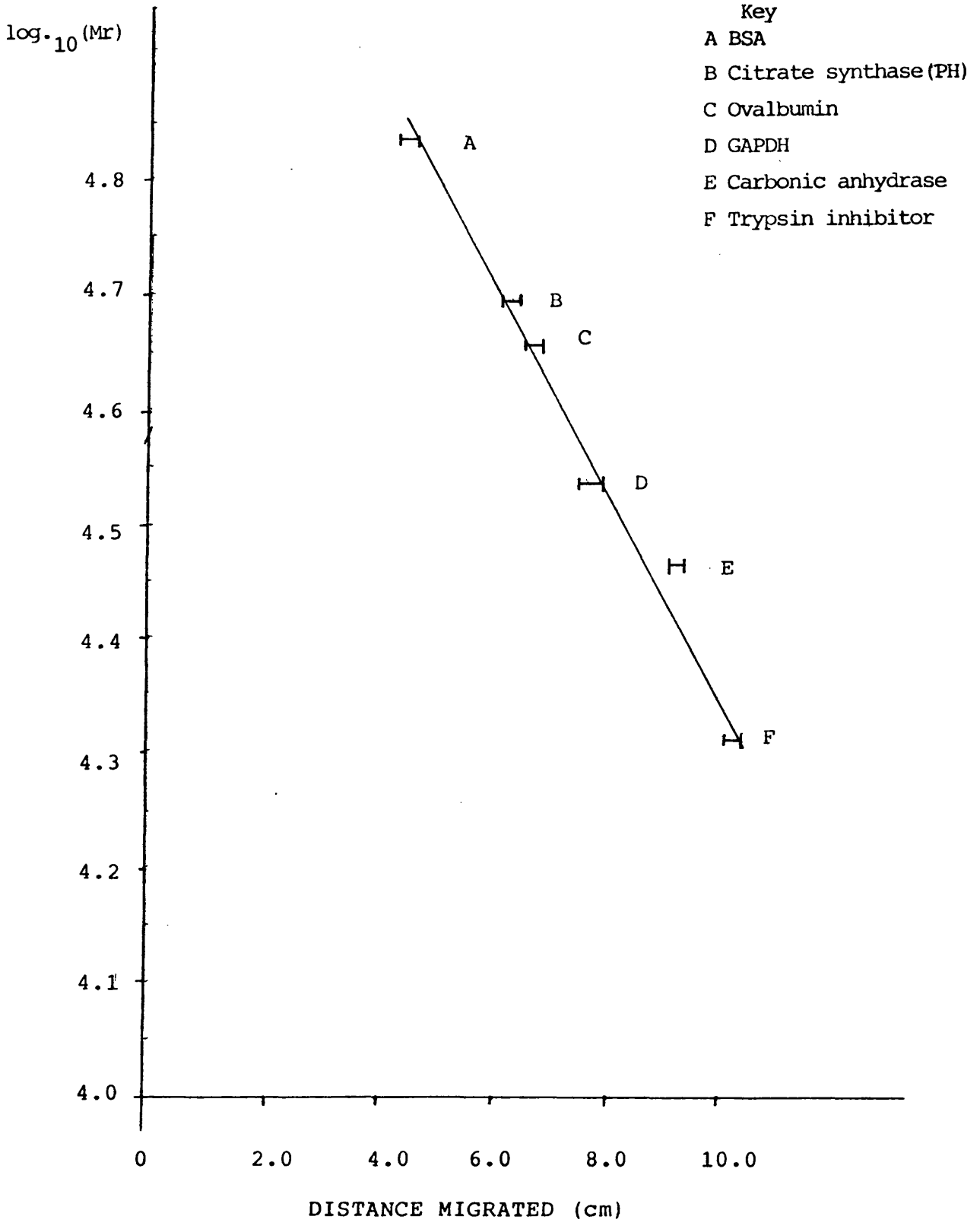
6.2.4. Substrate specificity of the purified enzyme.

Throughout the purification the relative rates of glucose and galactose oxidation with $NADP^+$ and NAD^+ were

Graph 6.2.3.1. Showing The Calibration Curve For The Superose 12 FPLC Gel Filteration Column.



Graph 6.2.3.2. Showing The Calibration Curve Obtained With The SDS PAGE Gels To Determine Subunit Structure.



monitored. The results are shown in table 6.2.4.1. .They show that each activity is conserved, with slight enrichment of galactose/$NADP^+$-dependant activity but a slight loss in glucose/NAD^+-dependant activity. The data tend to support the kinetic analysis described above, that is the conclusion that glucose dehydrogenase from Tp. acidophilum can accept both $NADP^+$ or NAD^+ to oxidise either D-glucose or D-galactose.

Mannose and glucose 6-phosphate were not oxidised by the purified enzyme.

6.2.5. Stability.

Since Tp. acidophilum glucose dehydrogenase is a thermostable enzyme, it was of interest to show how stable it was to storage and solvents.

6.2.5.1. Storage

Methanol-purified enzyme was incubated at 4°C and 37°C with and without substrates for 21days in 100mM Tris/HCl buffer, pH8.0. At 4°C, 90% the activity was recovered without substrates;however, at 37°C without substrates 60% of the activity was recovered compared to 0% with substrates.

6.2.5.2. Solvent.

To investigate the stability towards solvents, the enzyme was assayed under the standard conditions at 55°C, in the presence of various concentrations of methanol and acetone. The mesophilic enzyme rabbit muscle glucose 6-phosphate dehydrogenase was assayed at 37°C with the same

Table 6.2.4.1. Relative rates of oxidation throughout purification of glucose dehydrogenase.

The preparation of glucose dehydrogenase from Tp. acidophilum was assayed for activity with glucose , NAD^+, and $NADP^+$ and also galactose, NAD^+, and $NADP^+$.

PURIFICATION STAGE	% ACTIVITY OF PREPARATION			
	50MM GLUCOSE		50mM GALACTOSE	
	0.4mM $NADP^+$	5.0mM NAD^+	0.4mM $NADP^+$	5.0mM NAD^+
CRUDE	100	8.7	53	4.0
METHANOL	100	2.6	80	2.0
MONO Q	100	5.4	73	2.4
MONO P	100	2.5	69	1.2
SUPEROSE 12	100	4.6	87	2.0

concentrations of methanol. The data in figure 6.2.5.1. show that the thermostable enzyme retains half the initial activity at concentrations of about 55%(v/v) methanol and 45%(v/v) acetone in the cuvette, whereas the mesophilic enzymes possessed 50% activity at only 15%(v/v) methanol.

When glucose dehydrogenase was incubated in 100%(v/v) methanol for 30min at 20°C, a procedure which caused it to precipitate out, and then redissolved in 100mM Tris/HCl buffer, pH8.0, 100% of the activity was recovered, whereas when rabbit muscle glucose 6-phosphate dehydrogenase was treated under the same conditions no recordable activity was recovered. This property was used in the purification of the thermostable enzyme.

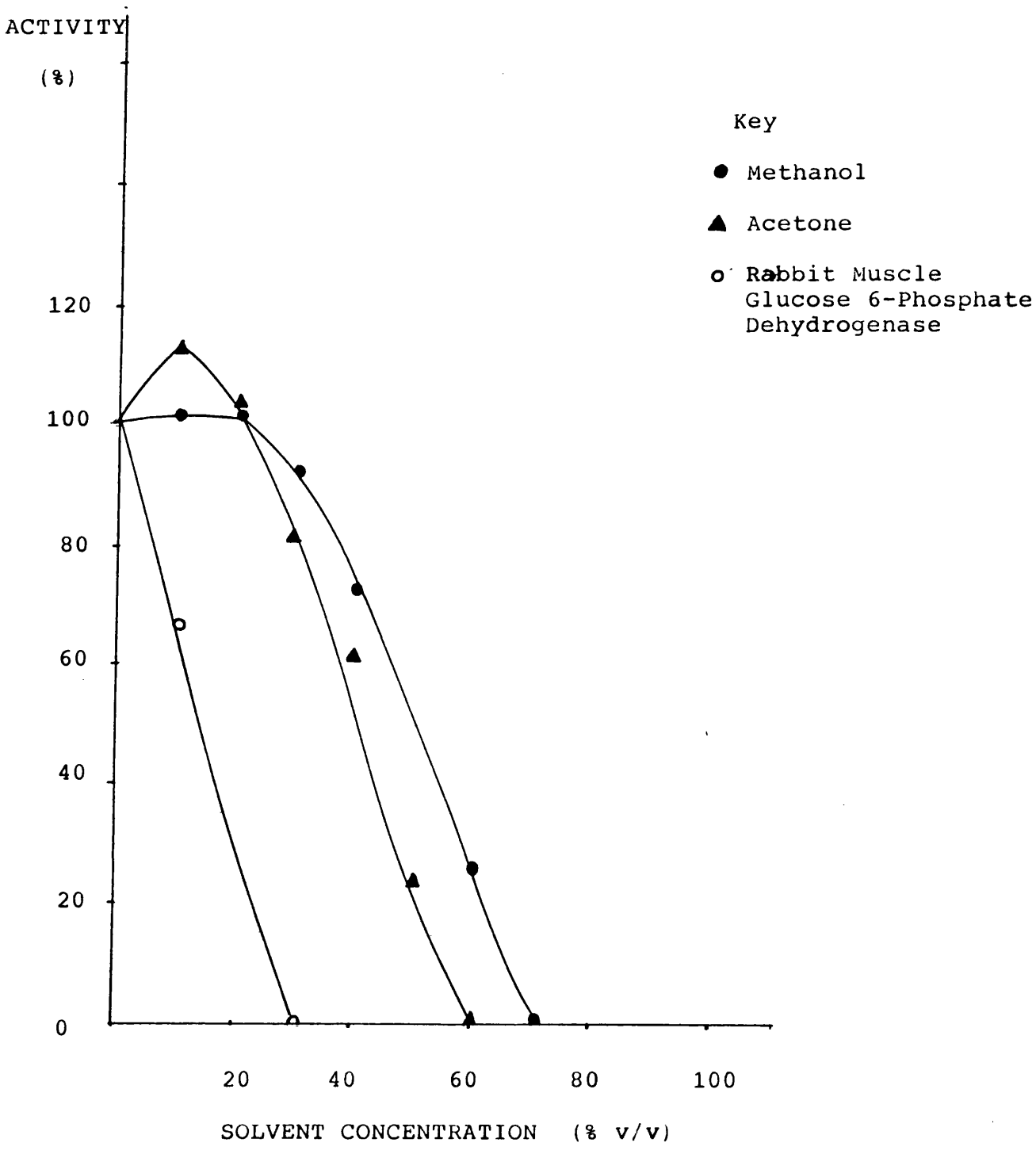
6.2.6. Effect of divalent metal ions on activity

The effect of Mg^{2+} and Ca^{2+} on the activity of glucose dehydrogenase was investigated. Purified enzyme was assayed with varying concentrations of $MgCl_2$, $CaCl_2$ and also EDTA and $MgCl_2$. The data in figure 6.2.6.1. show that both Ca^{2+} and Mg^{2+} inhibit the activity of Tp. acidophilum glucose dehydrogenase; 50% inhibition was found with 2.5mM of each ion. EDTA overcame the effects of Mg^{2+}, the enzyme retaining about 75% of its initial activity.

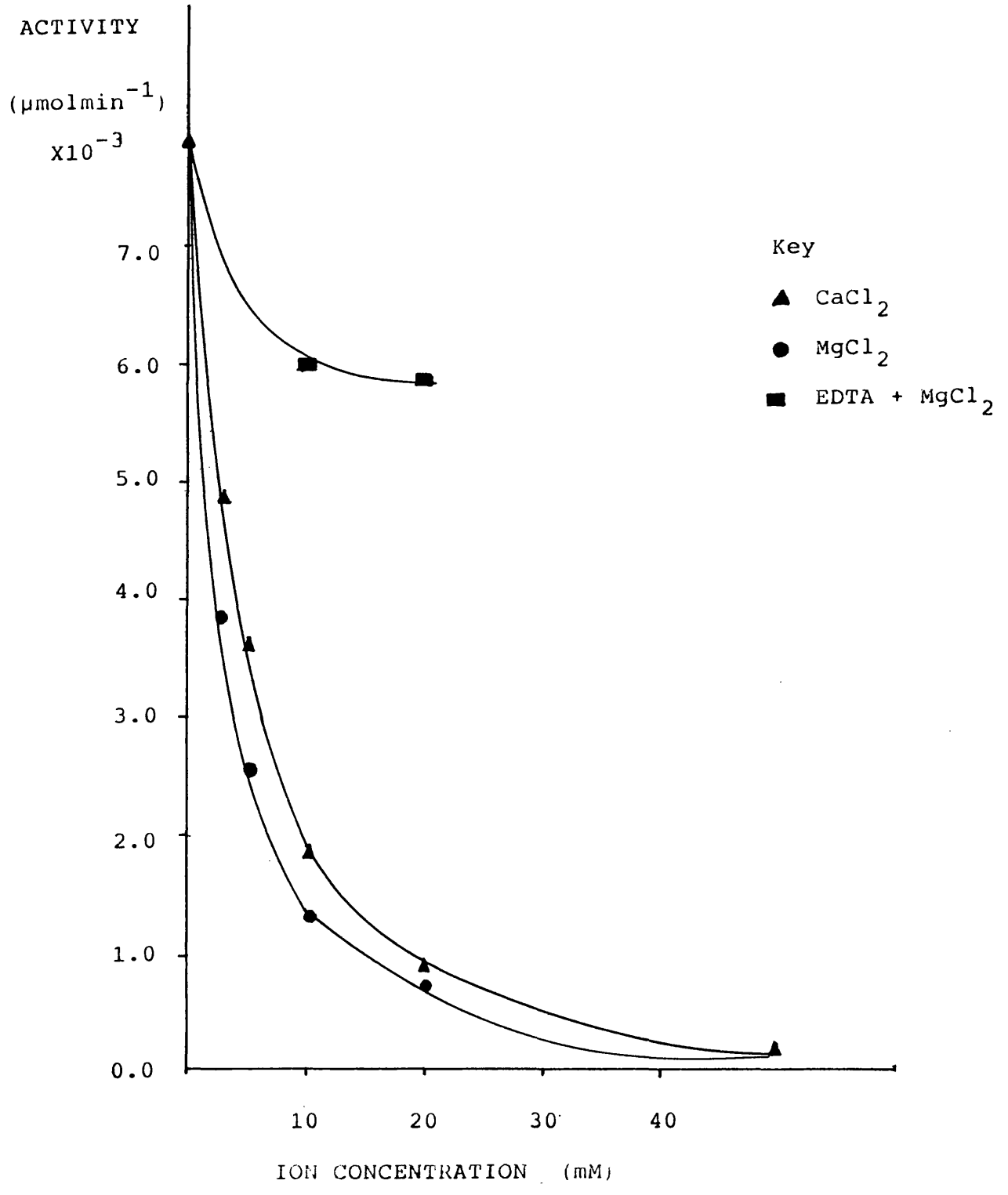
6.2.7. Reduction of gluconate by purified enzyme.

Purified enzyme was assayed as described in the methods section with gluconate and NADPH to follow the reaction;

Graph 6.2.5.1. The Effect on Activity by Methanol and Acetone on Glucose Dehydrogenase From Tp. acidophilum And Glucose 6-Phosphate Dehydrogenase From Rabbit Muscle.



Graph 6.2.6.1. Inhibition of Glucose Dehydrogenase Activity by $MgCl_2$ and $CaCl_2$.



D-gluconate + NADPH + $H^+ \longleftrightarrow$ glucose + $NADP^+$

The specific activity of the purified enzyme catalyzing this reaction was $0.285(\pm 0.05)\mu mol.min^{-1}mg^{-1}$ which is 1% of the rate found with the oxidation of glucose. This slow rate may be explained if $-gluconolactone is an intermediate in the reaction. As high temperature and alkaline pH will cause hydrolysis of the lactone, the intermediate concentration will be low, favouring the formation of gluconate.

6.3. Discussion.

The data provided by the purification and kinetic analysis of Tp. acidophilum glucose dehydrogenase are consistent with a dual-specific enzyme capable of oxidising glucose and galactose with both $NADP^+$ and NAD^+. The kinetic analysis shows competition between glucose and galactose, and between NAD^+ and $NADP^+$, suggesting that both members of each pair share the same active site. The observed relative rates of glucose and galactose oxidation facilitated by $NADP^+$ and NAD^+ did not vary significantly throughout the purification, suggesting dual specificity. The monosaccharide mannose, however was not oxidised at any stage.

Dual cofactor specificity has also been observed with the glucose dehydrogenases isolated from the thermoacidophilic archaebacterium S. solfataricus (Giardina et al., 1986) and the sporulating eubacteria Bacillus subtilis and B. cereus. As already discussed, this feature is uncommon in eubacteria and eukaryotic dehydrogenases but appears a more common feature in the thermoacidophilic archaebacteria.

It is also interesting to note that the Km for NAD^+ is more than 100 fold greater than the Km for $NADP^+$ in the Tp. acidophilum enzyme, a ratio which compares to that of 40 times in the S. solfataricus glucose dehydrogenase.

The S. solfataricus enzyme oxidises a wide range of substrates including mannose and galactose in an $NADP^+$ dependant reaction whereas the Tp. acidophilum enzyme is unable to oxidise mannose. Glucose dehydrogenases purified from other organisms show quite varing monosaccaride

substrate specificity suggesting that they possess different active sites.

Tp.acidophilum glucose dehydrogenase appears to be a dimer of Mr=70,000 which is much smaller than the tetramer of Mr=124,000 for the S. solfataricus enzyme described by Giardina et al. (1986). It also contrasts with those isolated from Gluconobacter sp. in which the enzyme is a tetramer of Mr=150,000, (Adachi et al.,1980), Corynebacterium sp. in which it is a single polypeptide of Mr=55,000 (Kobayashi & Honkoshi, 1980) and Bacillus sp. in which it is a tetramer of Mr=120,000 (Fujita et al.,1977). This again illustrates the diverse forms of the enzyme throughout the kingdoms with no particular type being associated with a group of organisms.

Tp. acidophilum glucose dehydrogenase is extremely stable to the organic solvents methanol and acetone, being reversibly denatured in concentrations in excess of 70%(v/v) The mesophilic glucose 6-phosphate dehydrogenase was irreversibly denatured under the same conditions. The observed solvent stability of the archaebacterial enzyme may be the result of properties which confer its thermostability. Similarly the enzyme purified from thermoacidophile S. solfataricus (Giardina et al., 1986) was shown to be stable to 50%(v/v) acetone and methanol for up to 24 hours at room temperature, and no appreciable loss of enzyme activity was observed with 2M urea and 0.05%(w/v) SDS.

Ca^{2+} and Mg^{2+} ions inhibited the enzyme from Tp.acidophilum, 20mM causing a 90% reduction of activity, an effect which

was overcome by 20mM EDTA. However the enzyme from S. solfataricus is activitated by both these ions when assayed with NAD^+ and only 5% of the maximum activity achieved with 20mM of the bivalent ion was recorded with 50mM EDTA. Thus divalent ions have completely opposite effect on these two archaebacterial glucose dehydrogenases suggesting again a contrasting structure.

Work continued on this enzyme by L.D.Smith,of the same laboratory , has successfully isolated the protein. The pure enzyme shows dual cofactor specificity, but a native molecular weight of 140,000.The cofactor specificity agrees with the work described here but the Mr does not, although similar techniques were used to determine its size. Perhaps Tp. acidophilum glucose dehydrogenase also exhibits an equilibrium of various molecular forms.

Concluding Remarks

Tp. acidophilum and S. solfataricus glucose dehydrogenase have both similar and yet contrasting properties. They both show dual specificity with the cofactors $NADP^+$ and NAD^+, and glucose and galactose, but differ in their ability to oxidise mannose. They also are both stable to temperature and organic solvents. However they appear to have different molecular weights and divalent ions have opposite effects on enzyme activity.The two archaebacteria have evolved two quite different enzymes that appear to serve in similar pathways of glucose catabolism, but still share some fundamental properties.

CHAPTER 7.

DISCUSSION.

In this final chapter,the conclusions drawn from the experimental work on Tp.acidophilum are discussed in relation to the results and theories of other workers. Firstly, the existence of the non-phosphorylated Entner-Doudoroff pathway is discussed in relation to the currently accepted phylogenic theories and this is then followed by a consideration of the properties of this novel pathway.

7.1. Phylogeny.

The phylogenetic trees of the archaebacteria based on 16S rRNA sequence analysis (Woese & Olsen, 1986) and rRNA/DNA hybridisation (klenk et al., 1986) show three main branches.Both analyses place Thermoplasmales and Sulfolabales in different branches, indicating that they are not closely related. Also both trees position Tp. acidophilum close to Halobacteriales, Methanococcales, and Methanomicrobiales, away from the other thermophiles. It is also interesting to note that, because thermophiles are found in each of the branches, thermophily is thought to be an ancient phenotype.

On the evidence presented in this thesis, Tp. acidophilum shares common metabolic characteristics with members of Sulfolobales. In both Sulfolobus solfataricus and Tp. acidophilum, the non-phosphorylated Entner-Doudoroff

pathway has been reported, although they differ in the catabolism of glyceraldehyde. An unspecified Entner-Doudoroff pathway has been reported in another Sulfolobales Sulfolobus brierleyi, again exhibiting a similarity with Tp. acidophilum. Another metabolic characteristic common to both genera is the synthesis of glycerol, as presented in this thesis in chapter 4. Similar enzymatic activities were identified in Tp. acidophilum and S.solfataricus also show similarities with respect to cofactor specificity, as presented in chapter 6. All these features suggest the two orders Thermoplasmales and Sulfolobales are metabolically similar.

The phylogenic trees based on gene sequence analysis are not questioned by this limited study. Conclusions drawn from the work described in this thesis must be consistent with the phylogenic trees described (figure 1.1.1 and 1.1.2) which place Tp. acidophilum remote from Sulfolobales. Considering this, possession of the non-phosphorylated Entner-Doudoroff pathway in Tp. acidophilum and S.solfataricus suggest that it is an ancient pathway, as it is present across a wide evolutionary divide. The route of glycerol synthesis which is common between the two genera could, by the same reasoning, be ancient.

The third phylogenetic branch is at present represented by the thermophilic order Thermococcales (figure 1.1.2.) which is placed between Thermoplasmales and Sulfolobales. It would be of considerable consequence to demonstrate the pathway of glucose catabolism in this order which would

add to the current information.

The modifed Entner-Doudoroff pathway has also been demonstrated in the Halobacteriales Halobacterium saccharovorum (Tomlinson et al.,1974). This is again an unusual pathway, however is not just restricted to the archaebacteria as it is found in a number of eubacteria. Tomlinson et al.(1974) found in addition to this pathway that pyruvate production from glucose did not have an absolute requirement for ATP. This observation suggests that this organism may also have a non-phosphorylated Entner-Doudoroff pathway.

As mentioned in the introduction no phosphofructokinase activity has yet been discovered in any archaebacterial species, making the existence of an Embden-Meyerhof pathway remote. This conclusion is challenged by NMR labeling study on Methanobacterium thermoautotrophicum (Evans et al. 1985,1986) which provides strong evidence for the catabolism via an Emben-Meyerhof pathway.

In conclusion, some members of the archaebacteria have evolved ways of catabolising glucose based on the Entner-Doudoroff pathway and not the more commonly encountered Embden Meyerhof pathway.

7.2. The Novel Pathway.

Having established the possibility that the non-phosphorylated pathway is ancient, we shall now consider its evolution with respect to the other pathways of glucose catabolism.

Glucose catabolism seems remarkably conserved throughout

the wide spectrum of organisms. This is no more apparent than with the metabolism of glyceraldehyde 3-phosphate to pyruvate (figure1.2.1.). This set of reactions forms part of the phosphorylated Entner-Doudoroff pathway and Embden-Meyerhof pathway, and is therefore found in virtually every organism capable of metabolising glucose. It has a net yield of two ATPs per triose-phosphate converted to pyruvate and is important for providing energy. The fact that this set of reactions is so widely found suggests that they were possessed by a common ancestor to most organisms. This assumes that they were not evolved separately. From the results described in this thesis, these reactions are absent from Tp. acidophilum, since it appears to catabolise glyceraldehyde to pyruvate via glycerate. However it does possess two enzymes enolase and pyruvate kinase, that are common to the pathway which is phosphorylated. Has Tp. acidophilum evolved a pathway from the complete sequence, hence losing the capability of metabolising glyceraldehyde 3-phosphate? Since it possesses enolase and pyruvate kinase it must have evolved from at least part of the sequence. If it evolved from the complete sequence it must have lost the ability of performing substrate level phosphorylation whilst oxidising the glyceraldehyde, which would, from an energetic standpoint, appear unlikely. This reasoning infers that Tp.acidophilum has evolved the ability to oxidise glyceraldehyde from an organism which did not possess the complete energy yielding sequence found in the majority of organisms.

Now we will consider the first three steps of the non-phosphorylated pathway. These are based on the Entner-Doudoroff pathway, which strongly suggests that it has evolved from a similar set of reactions. Since there is no net yield of ATP from the first three reactions in either the conventional or non-phosphorylated Entner-Doudoroff pathways there is no energetic disadvantage between them.

As already discussed, the non-phosphorylated pathway in Tp. acidophilum has no net yield of ATP. This compares with two ATPs per molecule of glucose as found with the Embden-Meyerhof and one ATP with the conventional Entner-Doudoroff. The pathway in Tp.acidophilum rather than being of an energy conserving role, may serve for the production of intermediates for metabolic activity, This would be the expected role of an ancient pathway.

In conclusion Tp.acidophilum possesses a pathway which is fundamentally different from other glycolytic pathways. This contrasting role and structure indicates an ancestry which may have branched from the early mainstream of organisms at an early stage of the evolutionary process.

6.3. The Non-Phosphorylated Pathway.

Thermoplasma acidophilum metabolises glucose by a non-phosphorylated pathway. This poses a question. How does this effect the diffusion of intermediates across the cell membrane? Davis (1958) in a paper entitled "The importance of being ionised", argued that organisms conserved these metabolites within their cell membranes by making them ionised

at cellular pH values. In pathways such as the Embden-Meyerhof this is achieved by the phosphorylation of all intermediates. Since the metabolites of the pathway in Tp. acidophilum are not phosphorylated, how are they prevented from being lost? This question may be answered by observing that glucose is oxidised to gluconate (which will be charged at the internal pH of pH 6.0) by the enzyme glucose dehydrogenase. Hence glucose will be trapped in the cell as gluconate, formed by the action of this highly active enzyme identified in this thesis. Not all the intermediates of the pathway are charged such as glyceraldehyde and glycerol. Diffusion of these compounds may be reduced by maintaining a low cellular concentration as a result of the flux through the pathway. This has to be proved.

It is interesting to note that a non-phosphorylated version of the Embden-Meyerhof pathway would lack charged intermediates, the first product being fructose and then glyceraldehyde. This may mean that a non-phosphorylated Embden Meyerhof pathway would be unfavourable and would be selected against in nature. This perhaps highlights the versatility and flexibility of the Entner-Doudoroff pathway as opposed to the Embden-Meyerhof sequence of reactions.

What might be the advantages for Tp. acidophilum in utilising the non-phosphorylated pathways? The answer may be the result of the thermal environment in which it lives. Certain phosphorylated compounds are unstable in

neutral conditions and high temperature (glyceraldehyde 3-phosphate is one such compound). This thermal liability may make it inefficient for Tp. acidophilum to utilise such intermediates. However it must be pointed out that other thermophiles, such as Bacillus stearothermophilus, which grow at 60°C, possess the Embden-Meyerhof pathway.

To sum up, the adoption of this unique pathway by Tp. acidophilum may be the result of the flexibility of the Entner-Doudoroff pathway over and above the Embden-Meyerhof pathway, and the product of the environmental constraints in which it exists.

7.4 Other Theories.

Searcy & Whatley (1984) consider Tp. acidophilum to be an ancestral candidate for the cytoplasmic/nuclear componant of eukaryotic cells. They arrived at this conclusion from the observation that its chromosomal proteins, cytoskeleton and resiratory chain components are all similar to eukaryotic cytoplasmic proteins (Searcy et al. 1981; Searcy 1982; Barnabas et al., 1982). From this hypothesis, they predict that the organism should have an Embden-Meyerhof pathway and not an Entner-Doudoroff pathway. Evidence provided in this thesis of a non-phosphorylated Entner-Doudoroff pathway contradicts their predictions. Also, the pathway of glycerol synthesis for lipids differs from the eukaryotic pathway is again contrary to Searcy's views.

However, it should be mentioned that the enzyme acetyl-CoA synthatase found in Tp. acidophilum which is ADP

dependant, has only been reported so far in one other organism, an eukaryote Entamoeba histolytica (Reeves et al.,1977). Rather than being of any great evolutionary significance, the similarity of the two enzymes may be the result of their metabolic role. In both organisms, the enzyme serves as an energy yielding function producing acetate, rather than consuming it as usually associated with the AMP-dependant acetyl-CoA synthatase. To conclude, the findings reported in this thesis do not support Searcy's hypothesis that Tp. acidophilum is related to the eukaryotic cytoplasmic/nuclear component.

7.5. Concluding Remarks

The majority of the biochemical features so far reported that have been used to compare the archaebacteria with the eubacteria and eukaryotes have been based on their molecular biological characteristics. It is hoped that this research has opened a new chapter in the comparative biochemistry of the metabolic pathways found in each of the three kingdoms, and laids the foundation stone for further enzymological studies in the archaebacteria.

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